Ampliación, mejora y aplicación de plataformas metabolómicas para la determinación de vitamina D y sus metabolitos

Expansion, improvement and application of metabolomic platforms for the determination of vitamin D and its metabolites

TESIS DOCTORAL
Antonio Mena Bravo
Córdoba, 2019

TITULO: *Ampliación, mejora y aplicación de plataformas metabolómicas para la determinación de vitamina D y sus metabolitos*

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UNIVERSIDAD DE CÓRDOBA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

AMPLIACIÓN, MEJORA Y APLICACIÓN DE PLATAFORMAS METABOLÓMICAS PARA LA DETERMINACIÓN DE VITAMINA D Y SUS METABOLITOS

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Los directores,

Fdo. **María Dolores Luque de Castro**
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Trabajo presentado para optar al grado de Doctor en Ciencias, Sección Química

El doctorando,

Fdo. **Antonio Mena Bravo**
Licenciado en Química

María Dolores Luque de Castro, Catedrática Emérita, y **Feliciano Priego Capote**, Profesor Titular, ambos del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por el licenciado químico ANTONIO MENA BRAVO**,** con el título "**Ampliación, mejora y aplicación de plataformas metabolómicas para la determinación de vitamina D y sus metabolitos**",

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, diciembre de 2018.

Fdo. María Dolores Luque de Castro Fdo. Feliciano Priego Capote

Mediante la defensa de esta Memoria se pretende optar a la mención de **Doctorado Internacional**, habida cuenta de que el doctorando reúne los requisitos exigidos para tal mención, a saber:

1. Informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:
   - Prof. Dr. Jean-Michel Kauffmann, Department of Research in Drug Development at the Faculty of Pharmacy, University Libre de Bruxelles, Belgium.
   - Prof. Dr. Oleg A. Mayboroda, Centre for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, Netherlands.
2. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país:
   - Prof. Dr. Roger Bouillon, Laboratory for Experimental Medicine and Endocrinology, University KU Leuven, Belgium.
3. La exposición y la defensa de parte de esta Tesis se realizarán en una lengua diferente a la materna: inglés.
4. Estancia de tres meses en un centro de investigación de otro país:
   - School of Pharmacy, University of Eastern Finland, Kuopio, Finlandia, bajo la supervisión del Prof. Dr. Seppo Auriola.

TÍTULO DE LA TESIS: Ampliación, mejora y aplicación de plataformas metabolómicas para la determinación de vitamina D y sus metabolitos

DOCTORANDO: Antonio Mena Bravo

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma)

El doctorando Antonio Mena Bravo mostró, desde su incorporación al grupo de investigación del que ha formado parte durante 5 años, un gran interés por la metabolómica clínica y por la instrumentación adecuada para obtener la información requerida en cada caso en estudio. Fruto de ello ha sido su excelente formación sobre vitamina D y sus metabolitos, de la que han resultado los artículos que componen la Memoria de la Tesis, además de los incluidos como anexos: todos ellos constituyen un aval patente de las mejoras indiscutibles que se han conseguido con el desarrollo de la investigación realizada. Resultado de la formación adquirida es también el interés de la firma Agilent Hispania (de la que se adquirió en su día la mayor parte de la instrumentación de la que dispone el grupo) por el doctorando para formar parte de su equipo técnico.

Los avances conseguidos con el desarrollo de la investigación recogida en esta Memoria se deducen del título de la tesis y son los siguientes:

El desarrollo de un método totalmente automatizado basado en una plataforma formada por una estación comercial de extracción en fase sólida conectada en línea con un cromatógrafo de líquidos y un detector de masas de triple cuadrupolo (SPE–LC–MS/MS QqQ) para la determinación en sangre de vitamina D (tanto de la forma D_2 como de la D_3) y sus principales metabolitos {monohidroxilados —25(OH)D_2 y 25(OH)D_3—, y dihidroxilados —1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, y 24,25$(OH)_2D_3$)}, con utilización de patrones marcados isotópicamente (SIL-ISs) y validación externa basada en el programa DEQAS. La posterior ampliación del método para abarcar la discriminación de los epímeros del monohidroximetabolito 25(OH)D_3 requirió un desarrollo cromatográfico bidimensional, que se validó mediante el programa DEQAS y con un material de referencia certificado de la NIST. Todo ello pone de manifiesto la solidez del estudio.

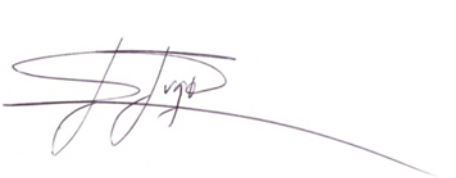
Para completar los aspectos relacionados con la aplicabilidad del método se realizó el estudio de la idoneidad del suero y/o el plasma para determinar los compuestos en estudio, del tipo de tubo para la recogida de la muestra y de la necesidad o no de desproteinizarla. Finalmente, un estudio de la estabilidad de los diferentes metabolitos cuando las muestras se sometieron a diferentes condiciones de almacenamiento (temperatura, ciclos de congelación/descongelación), y un estudio de liofilización, pusieron de manifiesto que es esta última forma la que permite una mejor conservación de los analitos.

La masiva aplicación del método a muestras de suero de diferentes centros de investigación (Instituto de Salud Carlos III, Hospital San Rafaelle, de Milán, Italia, etc.) de pacientes afectados por diferentes enfermedades (cáncer de mama, cambios postmenopáusicos, esclerosis múltiple, etc.) y el ajuste de los resultados a las hipótesis propuestas han puesto de manifiesto que el estudio se ha realizado con un gran rigor y que los resultados son de una enorme aplicabilidad en el área clínica.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 3 de diciembre de 2018

Firma de los directores



Fdo.: M. D. Luque de Castro

Fdo.: F. Priego Capote

INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES DE LA TESIS

TÍTULO DE LA TESIS: AMPLIACIÓN, MEJORA Y APLICACIÓN DE PLATAFORMAS METABOLÓMICAS PARA LA DETERMINACIÓN DE VITAMINA D Y SUS METABOLITOS

Publicaciones	FI	Decil/cuartil
Sweat: a sample with limited present applications and promising future in metabolomics. **J. Pharm. Biomed. Anal.** 2014; 90, 139–147	2.979	Q1 16/18 Analytical Chemistry
Quantitative analytical method to evaluate the metabolism of vitamin D. **Clin. Chim. Acta** 2015; 442, 6–12.	2.799	Q1 7/7 Medical Laboratory Technology
Study of blood collection and sample preparation for analysis of vitamin d and its metabolites by liquid chromatography–tandem mass spectrometry. **Anal. Chim. Acta** 2015; 879, 69–76.	5.123	D1 8/81 Analytical Chemistry
Pharmacokinetics and pharmacodynamics of ramipril and ramiprilat after intravenous and oral doses of ramipril in healthy horses. **Vet. J.** 2016; 208, 38–43	1.773	Q1 26/140 Veterinary Sciences
Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D3. **J. Chromatogr. A** 2016; 1451, 50–57.	3.716	Q1 13/81 Analytical Chemistry
Pharmacokinetic/pharmacodynamic modeling of benazepril and benazeprilat after administration of intravenous and oral doses of benazepril in healthy horses. **Res. Vet. Sci.** 2017; 114, 117–122.	1.616	Q1 31/140 Veterinary Sciences
Serum 25-hydroxyvitamin D and breast cancer risk by pathological subtype (MCC-Spain). **J. Steroid. Biochem. Mol. Biol.** 2018; 182, 4–13.	4.095	Q1 69/293 Biochemistry & Molecular Biology
Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. **Food Chem.** 2018; 266, 192–199.	4.964	D1 5/72 Applied Chemistry
Oleocanthalic acid, a chemical marker of olive oil aging and exposure to high storage temperature with potential neuroprotective activity. J. Agric. Food Chem. 2018; 66(28), 7337–7346.	3.412	D1 2/57 Agriculture, multidisciplinary
Determination of primary fatty acid amides in different biological fluids by LC–MS/MS in MRM mode with synthetic deuterated standards: Influence of biofluid matrix on sample preparation. **Talanta** 2019; 193, 29–36.	4.244	Q1 10/81 Analytical Chemistry

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OBJETIVOS

OBJECTIVES

El **objetivo general** de la investigación que ha dado lugar a esta Memoria de Tesis fue optimizar, validar y aplicar un método para la determinación de vitamina D y sus metabolitos en sangre (suero y plasma). Los analitos a estudiar abarcaron la vitamina D (en las formas D_2 y D_3) y sus principales metabolitos mono y dihidroxilados. Se pretendió soportar el método en: (i) la completa automatización del proceso analítico (para un funcionamiento ininterrumpido y sin atención del operador durante 24 h/día, evitando o minimizando así la sobrecarga de muestras en el laboratorio), además de una adecuada validación. (ii) La multideterminación, que abarcara tantos analitos como fuera posible para obtener información cuantitativa del metabolismo de la vitamina D. (iii) Un estudio en profundidad de la información básica sobre las primeras etapas del método para la cuantificación de la vitamina D y sus metabolitos y su impacto en las propiedades analíticas del mismo: la selección del tipo de muestra derivada de la sangre total (suero o plasma) y del contenedor de muestra y, especialmente, el procedimiento óptimo de preparación de la muestra. (iv) La suficiente información sobre la estabilidad de la muestra para evaluar el efecto de la temperatura de almacenamiento durante un periodo establecido de dos meses en las propiedades analíticas del método en función del/los analito/s a determinar. (v) La aplicación masiva a muestras procedentes de diferentes centros de investigación y de pacientes con diferentes enfermedades, para poner así de manifiesto las fortalezas y debilidades del método y ayudar a conocer los cambios que se producen en los metabolitos a causa de enfermedades concretas.

Este ambicioso objetivo se consiguió paso a paso al ir alcanzando los siguientes **objetivos específicos**:

(i) Automatizar y validar un método basado en el acoplamiento en línea de una estación de extracción en fase sólida (SPE) a un cromatógrafo de líquidos conectado a un espectrómetro de masas en tándem (LC–MS/MS) para la determinación en suero de vitamina D (en las formas D_2 y D_3) y sus

principales metabolitos monohidroxilados (25-hidroxivitamina D_2 [$25(OH)D_2$] y 25-hidroxivitamina D_3 [$25(OH)D_3$]) y dihidroxilados (1,25-dihidroxivitamina D_2 [$1,25(OH)_2D_2$], 1,25-dihidroxivitamina D_3 [$1,25(OH)_2D_3$] y 24,25-dihidroxivitamina D_3 [$24,25(OH)_2D_3$]) constituyó un objetivo específico clave. El uso de estándares internos marcados isotópicamente (SIL-ISs) y la validación externa de acuerdo con el programa de validación "vitamin D External Quality Assurance Scheme (DEQAS)" contribuyeron a dar un soporte sólido al método, tal como recoge el Capítulo 1.

(ii) Ampliar el número de metabolitos determinados fue el objetivo específico del que resultó la discriminación de los epímeros $25(OH)D_3$, además de mantener el resto de los hidroximetabolitos. La cromatografía de líquidos bidimensional (LC×LC) con una etapa previa de SPE y la subsiguiente detección mediante MS/MS hicieron posible la consecución de este objetivo: un método ampliado y validado mediante aplicación a muestras del programa DEQAS y a un material de referencia certificado (CRM) del NIST; todo lo cual constituye el Capítulo 2.

(iii) Conocer la mejor muestra derivada de sangre total (suero o plasma) y el mejor procedimiento de preparación de la muestra fue el tercer objetivo específico, los resultados del cual conforman el Capítulo 3. Se utilizaron diferentes tubos de recogida, con y sin recubrimiento interno por gel, para obtener el suero y el plasma. Por otra parte, la información necesaria sobre la etapa de preparación de la muestra se obtuvo comparando cómo afecta a cada uno de los analitos la desproteinización o la SPE previas al análisis mediante LC–MS/MS.

(iv) Estudiar la estabilidad de los analitos de las muestras de suero sometidas a diferentes condiciones de conservación durante dos meses fue otro de los objetivos específicos. El estudio estadístico de los datos

obtenidos en el seguimiento mediante LC–MS/MS permitió conocer la magnitud de los cambios experimentados por cada uno de los compuestos en estudio, con especial énfasis en los metabolitos. Un logro interesante de este estudio fue la comprobación de que la estabilidad de los analitos se mantiene de forma general cuando las muestras se liofilizan. Todos estos aspectos constituyen el Capítulo 4.

(v) Validar el excelente comportamiento del método fue el último de los objetivos específicos de la Tesis, al cual el estudiante le dedicó un largo periodo de tiempo. Algunos de los ejemplos de aplicación del método por el doctorando son los siguientes: análisis de 546 muestras de suero de una cohorte de mujeres con cáncer de mama y 558 mujeres control (del proyecto MCC del Instituto de Salud Carlos III, Madrid); 1472 muestras de suero de una cohorte de mujeres pre- y postmenopáusicas (proyecto del mismo instituto); 314 muestras de suero de pacientes con esclerosis múltiple (proyecto sobre vitamina D y esclerosis múltiple en pacientes con síndromes clínicamente aislados, del Hospital San Rafaelle de Milán, Italia). Los datos analíticos han sido y están siendo en la actualidad usados con éxito por los participantes en los proyectos para la obtención de resultados que soporten o destruyan las hipótesis propuestas. Algunos de los datos obtenidos en los análisis realizados se han tratado desde el punto de vista analítico–quimiométrico y han dado lugar al Capítulo 5, cuyo contenido se ha enviado a la revista Journal of Steroid Biochemistry and Molecular Biology para su publicación. El resto de los resultados de los análisis masivos de muestras clínicas se resumen como Capítulo 6.

El **objetivo final** de una Tesis Doctoral es la formación del futuro doctor, que en este caso ha incluido el máster en "Química fina", en el que el doctorando ha desarrollado los cursos correspondientes. En paralelo con la investigación que constituye la parte experimental de la Memoria de Tesis, el doctorando ha

conseguido una formación más amplia con el desarrollo de las actividades que de forma sucinta se recogen como anexos:

Anexo I: Revisión bibliográfica sobre el papel del sudor como muestra analítica, una de las primeras publicadas en esta área.

Anexo II: Publicaciones derivadas del proyecto MCC.

Anexo III: Investigación realizada en colaboración con el Departamento de Farmacología, Toxicología y Medicina Legal y Forense de la Facultad de Veterinaria de la UCO.

Anexo IV: Investigación realizada en colaboración con el Departamento de Agronomía de la Facultad de Ciencias de la UCO.

Anexo V: Investigación realizada en colaboración con el Laboratorio de Farmacognosia y Química de los Productos Naturales de la Facultad de Farmacia de la Universidad de Atenas.

Anexo VI: Investigación realizada en colaboración con otros miembros del grupo al que pertenece el doctorando.

Anexo VII: Comunicaciones orales o en carteles en 7 congresos (3 nacionales y 4 internacionales).

The **general objective** of the research that has given place to this PhD-Book was to optimize, assess and apply a method for the determination of vitamin D and its metabolites in blood (serum and plasma). The analytes encompass vitamin D (both D_2 and D_3 forms) and its main mono- and dihydroxymetabolites. The main pillars to support the method were: (i) full automation of the overall analytical process — to work unattended for long time intervals able to establish 24-h working days, thus avoiding or minimizing sample overload—, and sufficient validation of the proposed method. (ii) Multidetermination, encompassing as many analytes as possible to obtain quantitative information of vitamin D metabolism. (iii) In-depth study of basic information on the first steps of the method for quantitation of vitamin D and its metabolites and their contribution to the analytical features of the method: selection of the type of blood-derived sample (serum or plasma) and the sample container, and specially, the optimal sample preparation procedure. (iv) Enough information on sample stability to evaluate the effect of storing temperature for a 2-month preset period on the analytical features of the method. (v) Massive application to samples from different research centers and patients with different diseases, thus checking the strengths and weaknesses of the method and helping to know the changes in metabolites owing to given diseases.

This ambitious objective was achieved step by step by reaching the following **specific objectives**:

(i) To automate and validate a method based on the on-line coupling of an SPE station to a liquid chromatograph–tandem mass spectrometer (LC–MS/MS) arrangement for in serum determination of vitamin D (both D_2 and D_3) and its main monohydroxylated metabolites (25-hydroxyvitamin D_2 [$25(OH)D_2$] and 25-hydroxyvitamin D_3 [$25(OH)D_3$]), and dihydroxylated metabolites (1,25-dihydroxyvitamin D_2 [$1,25(OH)_2D_2$], 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$]) constituted a key specific objective. The use of stable

isotopically labeled internal standards (SIL-ISs) and external validation according to the vitamin D External Quality Assurance Scheme (DEQAS) contributed to giving a solid support to the method, as shown in Chapter 1.

(ii) To expand the number of metabolites to be determined was the specific objective that resulted in discrimination of 25(OH)D_3 epimers, in addition to the rest of hydroxymetabolites. Two-dimensional liquid chromatography (LC×LC) with a previous SPE step and subsequent MS/MS detection allowed consecution of this objective that resulted in an expanded method, then validated by application to samples from the DEQAS program and a certified reference material (CRM) from the NIST, as shown in Chapter 2.

(iii) To know the best blood-derived sample (serum or plasma) and the best sample preparation procedure constituted the third specific objective, the results of which give place to Chapter 3. Different types of collection tubes, with or without coating gel, were used to obtain serum or plasma. On the other hand, sample preparation by deproteination or solid-sample extraction (SPE) prior to LC–MS/MS analysis and statistical study of how the different conditions affect to each of the target analytes provided the required information.

(iv) To study the stability of analytes of blood derived samples (serum) under different storage conditions for two months was another specific objective. The statistical study of the data obtained by LC–MS/MS monitoring allowed establishing the significance of the changes experienced by each of the target compounds, with especial emphasis on metabolites. An interesting achievement of this study was to check the stability of the analytes when the samples were lyophilized. All these aspects constitute the subject of Chapter 4.

(v) To assess the excellent performance of the method was the last specific objective of the Thesis, to which a significant period of time was devoted by the PhD-student. Thus, 546 serum samples from a cohort of women with breast cancer and 558 control women (project MCC, Health Institute Carlos III, Madrid); 1472 serum samples from a cohort of pre- and post-menopausal women (project from the same institute); 314 serum samples from patients with multiple sclerosis (project vitamin D and multiple sclerosis in patients with clinically isolated syndromes, San Rafaelle Hospital, Milan, Italy) are some examples of the experience of the PhD-student in applying the developed method. The analytical data have been, and are at present, successfully used by the projects participants to obtain results that support or bring down given hypotheses. Some of the data obtained in the analyses have been treated from the analytical–chemometric point of view giving place to Chapter 5, the content of which has been sent to Journal of Steroid Biochemistry and Molecular Biology for publication. The rest of the results from the massive analysis of clinical samples are summarized as Chapter 6.

The formation of the future PhD, **final objective** of a Doctoral Thesis, has included the master on "Fine Chemistry", in which the PhD student developed the mandatory courses. In parallel to the research that constitutes the experimental part of the PhD-book, a wider formation of the PhD student has been sought by development of other activities summarized as annexes:

Annex I: Review on the role of sweat as analytical sample, one of the first published in this field.

Annex II: Publications from the MCC project.

Annex III: Research developed in collaboration with the Department of Pharmacology, Toxicology and Legal and Forensic

Medicine, Faculty of Veterinary, UCO.

Annex IV: Research developed in collaboration with the Department of Agronomy, Faculty of Sciences, UCO.

Annex V: Research developed in collaboration with the Laboratory of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens.

Annex VI: Research developed in collaboration with other members of our research group.

Annex VII: Oral and poster communications in 7 meetings (3 national and 4 international meetings).

INTRODUCCIÓN

INTRODUCTION

1. Metabolomics: the key discipline for vitamin D research

1.1. Definition of terms

The late twentieth century, more than three decades ago, was notorious for revolutionizing the life sciences with the emergence and subsequent expansion of the disciplines encompassed under the terminology "omics" which derived from the Latin suffix ‘‘ome’’ meaning mass or many.

Omics studies involve the measurement of large numbers of parameters, typically genes (genomics), mRNA (transcriptomics), proteins (proteomics), or metabolites (metabolomics), resulting in primary omics. The measure of specific families such as lipids, glycans, toxics, foreign substances, or even interactions among them, provides the secondary omics (lipidomics, glycomics, toxicomics, xenometabolomics, and interactomics, respectively), all them as parts of primary omics.

The term metabolome and the derived omics were not used until 2002, when Fiehn defined metabolomics as a global and comprehensive analysis in which all metabolites of a biological system are identified and quantified [1]. However, the term metabolome first appeared in the literature in 1998, when Oliver *et al.* [2] measured the change in the relative concentrations of metabolites as the result of deletion or overexpression of a gene. In the last decade, other ways have appeared in the literature to define and classify metabolomic studies. Beyoğlu and Idle (2013) combined all of them in a broad definition of the discipline as "the global and unbiased study of small molecules (<1 kDa) in a biofluid, tissue, organ or organism" [3].

The information provided by metabolomics would lead to the complete metabolome, which is defined as the quantitative complement of low-molecular-weight metabolites (thus excluding proteins) present in a biological fluid, cell or

organism under a given set of physiological conditions or under different perturbations (*e.g.*, genetic variations, pathological states or responses to external stimuli) [4]. The metabolome, and therefore the metabolic state of a living organism, can be affected by various intrinsic factors (age, health status, reproductive status, etc.) and extrinsic factors (nutrients, artificial compounds such as pesticides, fertilizers, environmental pollutants, etc.). Therefore, the study of the metabolome is of great importance to know the state of an organism and how it is affected by the environment. In the case of cells and tissues, the metabolome comprises the endometabolome (all intracellular metabolites) and exometabolome (all metabolites that are excreted to the growth medium or extracellular fluid).

On the other hand, metabolism is an extensive network of metabolic reactions where the products of one reaction are the reactants of a subsequent reaction. Therefore, a metabolite is defined as "any intermediate or end product of metabolism, usually restricted to small molecules that are not genetically encoded". Depending on the metabolic pathways in which the metabolites are involved and their function, they can be classified into primary metabolites (those that are directly involved in the growth, development and reproduction of the organism) or secondary metabolites (those no directly involved in the processes of growth, development and reproduction, but generally they have a key biological function as, for example, defense against predators, parasites or diseases, competition among species, facilitating reproduction processes or in cellular signaling mechanisms).

In the great diversity of chemical structures in the metabolome, there are endogenous and exogenous metabolites; the former (including amino acids, organic acids, nucleic acids, fatty acids, sugars, vitamins, cofactors, pigments, antibiotics, etc.) are naturally produced by an organism, and the latter (such as drugs, environmental toxins, food additives, and other xenobiotics) come from the

interaction with the outside.

1.2. Relationship of metabolomics with other great omics and secondary omics

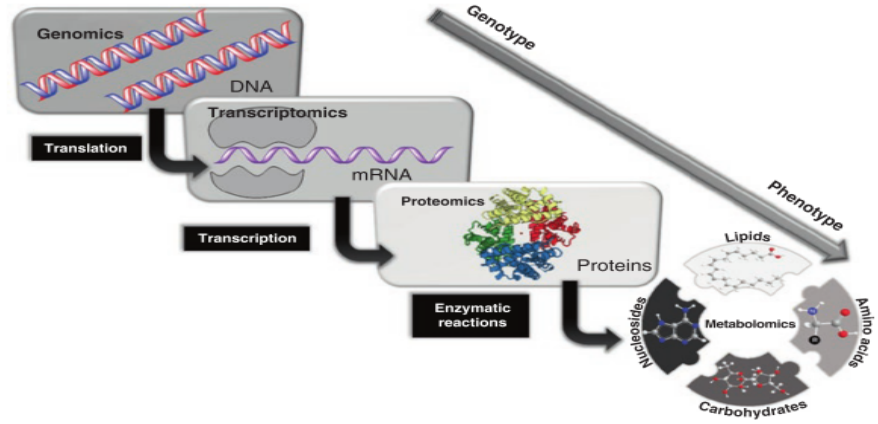
The structure and dynamics of cells and organisms functions, rather than the characteristics of isolated parts of them, must be examined to understand biology at the system level. The integration of the data from the different omics constitutes what was known as systems biology, term coined by Nicholson and Wilson [5] to better understand the functioning of a given biological system. At present, systems biology is more frequently known as integrative omics [6].

Figure 1 schematizes both the traditional central dogma of molecular biology, where the flow of information goes from genes to transcripts to proteins, finishing with the site where enzymes act on metabolism (Figure 1.A), and the omics organization (Figure 1.B), where the flow of biological information ends in the metabolites and is finally reflected in the phenotype.

A fundamental aspect that differentiates systems biology from the central dogma of molecular biology is that the latter is governed by unidirectionality in the expression of information from the genes of a cell or organism (*viz.*, DNA) to its transcription at mRNA and its translation to proteins that catalyze metabolic reactions, as shows Figure 1.A.

The metabolome is the level of the functional cascade that best reflects the physiological state, being not only the most sensitive to any change, either internal or external, but its ultimate expression, since the metabolites are the real active regulating agents of homeostasis [7]. Hence, metabolomics is one of the most powerful bioanalytical strategies as it allows obtaining a picture of the metabolites of an organism in the course of a biological process, being considered as a phenotyping tool, thus justifying its usefulness.

A)



B)

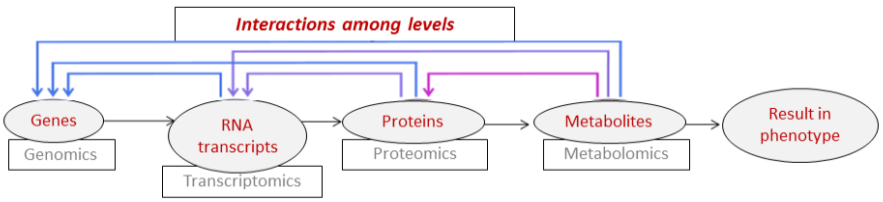


Figure 1. A) Traditional central dogma of molecular biology. B) General scheme of systems biology.

In contrast to proteins or genes, which are activated by post-translational or epigenetic modifications, respectively, metabolites result from cellular activity and serve as direct indicators of the biochemical activity of the cell in each time. In fact, physiological changes resulting from gene deletion or overexpression are amplified through the hierarchy of the transcriptome and proteome and are, therefore, more easily measurable through the metabolome, even when changes in metabolic fluxes are negligible [8]. Thus, small changes in the expression level of individual proteins have large effects on the concentrations of intermediary metabolites. As a result, the metabolome is more sensitive to overall perturbations than the transcriptome or the proteome, because the activity of metabolic

pathways is reflected more directly in the concentrations of metabolites than in those of the relevant enzymes or, indeed, those of their encoding mRNAs [9].

In brief, metabolomics has been developed into a more integrative approach than other omics approaches. Thus, it is not surprising that metabolic studies are increasingly being used in clinical diagnosis since the metabolic profile closely reflect the total cellular state. Serve as a measure of the relevance of the metabolomics a quote from James Watson, Nobel Laureate for the discovery of the DNA structure, who, in a recent interview, said: "If I had to do a doctorate right now, I would do it on metabolomics" [10]. Thus, the use of metabolomics has grown exponentially in the last decade. Nowadays, metabolomics is a mature discipline with a status similar to that of other major omics due to the relevance of the metabolome in the biological context. Figure 2 shows, in a comparative way, the evolution of the publications on the great omics including 2017.

Despite the great interest shared by many branches of biology and biotechnology in this new omics science, it must be admitted that metabolomics has not evolved as rapidly as genomics or proteomics. This is due to several aspects that are determined by the physicochemical characteristics of the small organic molecules. Unlike genes, mRNAs and proteins, all of which are biopolymers that encode information from a sequence of well-known monomers (or residues) —*e.g.*, nucleotides and amino acids—, the metabolites are chemical entities that do not originate from a transfer of information between residues within the cell. The great success in the characterization of genes, mRNA and proteins is a direct consequence of technologies and bioinformatics tools capable of amplifying and subsequently characterizing the sequence of nucleotides and amino acids, respectively, in these biopolymers. Metabolomics, however, aims to detect, quantify and elucidate the structure of the metabolites characterized by a great physicochemical diversity in their molecular structures [11].

The great diversity of molecules is reflected in a wide range of polarities,

molecular weights, functional groups, stability and chemical reactivity, among other key properties [12,13].

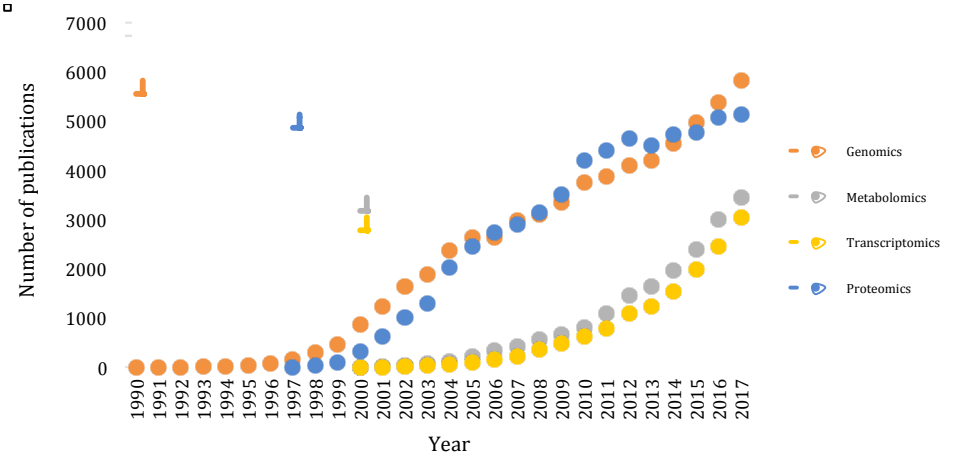


Figure 2. Evolution of the number of publications on the great omics since 1990 to 2017 (data collected from Scopus database).

This leads inevitably to use multiple platforms and analytical configurations that maximize the coverage of the analyzed metabolome, something that does not occur in genomics or proteomics experiments. However, metabolomics presents positive characteristics that promote its use and establish differences with other omics, such as:

-The existence of robust and stable analytical platforms for the analysis of a significant number of metabolites, as a result of research in this area during the last two decades of the twentieth century, prior to the appearance of omics as such.

-The analytical platforms used in metabolomics studies characterized by excellent analytical and biological reproducibility with a low cost of analysis per sample, as opposed to the costs of any other major omics application. This fact facilitates application to a high number of samples, and extensive studies in cohorts with a significant biological variability; thus providing wider and well-supported information.

-There are fewer metabolites than genes or proteins. According to a recent study, the predictable number of metabolites in humans is about 40.000 (29.000 of them endogenous [14]), while the number of genes, transcripts and proteins can reach 25.000, 300.000 and 1 000.000, respectively [15]. In the case of genomics, the inclusion of gene modifications —the subject of epigenomics— greatly increases the number of components [16]. Therefore, metabolomics studies lead to a reduction in the complexity of the data, thus simplifying the analysis and increasing the probability of detecting significant changes that reflect the biological problem under study [17].

- The technology involved in metabolomics is generic, as a given metabolite (unlike a transcript or protein) is the same in every organism that contains it [18].

- The ability to integrate data obtained with different techniques (integrated metabolomics) [19] applied to samples from different sources is other of its advantages.

- One other benefit is that, although some environmental perturbations or genetic manipulations may not cause changes at the transcriptomics and proteomics levels, they will have significant effects on the concentrations of numerous individual metabolites due to the “downstream” character of metabolomics.

Negative or unsolved aspects of metabolomics that make it more complex than other omics are the following:

- The necessity for multiple analytical platforms as a result of the enormous variety of metabolites that ranges from non-polar lipids to ionic compounds; from relatively big non-polar molecules such as cholesterol to very small ions such as ammonium.

- The concentrations at which the metabolites appear in the organisms cover more than nine orders of magnitude (from picomol to millimol) and make necessary the application of very different techniques both for sample preparation and for analysis of the target metabolites, with the researcher's experience playing a key role in selecting the most suitable as a function of both the nature of the metabolites to be determined and that of the sample matrix.

- The absence of enough standardized methods, as a consequence of being the youngest of the great omics.

Metabolomics can be applied to almost any type of matrix and can be focused on a multitude of case studies. Thus, the wide thematic diversity in metabolomics has led to a series of subdisciplines that, depending on their extension, can be classified as primary or secondary. Among the former are lipidomics, xenometabolomics, clinical metabolomics and nutrimetabolomics or nutritional metabolomics. Secondary subdisciplines of metabolomics are pharmaco-metabolomics, environmental metabolomics, cardiometabolomics, secretomics, cosmetobolomics, microbial metabolomics and many others that have emerged and will continue to emerge in view of the importance of the applications of metabolomics in new sectors.

Clinical metabolomics is aimed at evaluating the health status of an individual as

well as the risk of disease by any of the different metabolic analysis of biofluids or tissues, which are influenced by their genetic, epigenetic, environmental exposures, diet and behavior.

1.3. Metabolomics strategies

Metabolomics analysis encompasses different strategies the nature of which depends on the objective of the study and previous knowledge of the biological problem [20].

-*Targeted analysis*, defined as a quantitative analysis (concentrations are determined) or semiquantitative analysis (relative signals are registered) of a few metabolites and/or substrates of metabolic reactions that might be associated to common chemical classes or linked to selected metabolic pathways [21]. In this metabolomics approach there is a previous knowledge of the metabolites involved in the biological process under study. Therefore, selection of the most appropriate analytical technique and the best sample preparation as a function of the nature and abundance of the target metabolites, looking for sufficient sample cleanup and preconcentration, typically using solid-phase extraction (SPE) or liquid–liquid extraction (LLE).

A mass spectrometer is the detector of choice —particularly the triple quadrupole (QqQ) approach— in targeted metabolomics because of its sensitivity and selectivity. This was the strategy used and the instrument selected to develop the research that constitutes the present Thesis-Book.

-*Untargeted analysis,* based primarily on the qualitative or semiquantitative analysis of the largest possible number of metabolites from a diversity of chemical and biological classes contained in a biological specimen [22]. Detection of this wide range of metabolites can be done through a single analytical platform or a combination of complementary platforms, mainly based on nuclear magnetic

resonance (NMR) or mass spectrometry (MS), along with gas chromatography (GC), liquid chromatography (LC) or, less commonly, capillary electrophoresis (CE). In these studies, the relative concentrations of the analyzed metabolites are generally calculated and their variations between two or more system situations are studied. In untargeted approaches, the sample preparation step kept as simple as possible to obtain the widest metabolite coverage. Non-selective sample pretreatments such as protein precipitation (PP) for plasma/serum and simple dilution for urine samples (referred to as dilute-and-shoot) are generally used [23,24]. Besides, the data set obtained by applying these methods is very extensive, so it requires treatments with advanced chemometric approaches for conversion into manageable signals and, finally, interpretable results. The signals require annotation using either available experimental libraries, structural elucidation by *in silico* fragmentation tools or experimental identification. Thus, this strategy allows identification of new metabolites involved in metabolic pathways and, therefore, the knowledge of these routes. This strategy was not required for development of the planned research; nevertheless, the formation of the PhD student involved training on this strategy by using chromatographic equipment coupled to quadrupole time-of-flight (QTOF) detectors.

-*Metabolomics fingerprinting*, defined as a high throughput, fast methodology for analysis of biological samples that provides fingerprints for sample classification and screening [25]. Fingerprinting is not focused on particular metabolite(s); therefore, it allows discovery of novel metabolic pathways disturbed by the disease or preset stimulus.

-*Metabolomics footprinting*, term proposed in 2005 by Kell *et al.* to refer to the study of metabolites in extracellular fluids, also known as exometabolome or secretome [26]. As in the previous strategy, the purpose is mainly to obtain information for classification of groups of samples or for screening.

Each of these strategies has its own inherent advantages and disadvantages, but they can be highly complementary in combination.

The detection techniques used also depend on the chosen strategy. For example, to obtain a metabolomic fingerprint the detection technique should allow direct and rapid analysis of the sample. NMR, MS (depending on the complexity of the sample) and, to a lesser extent, infrared and Raman spectroscopies, are mainly used in this context. A separation technique is generally applied in both targeted and untargeted analysis prior to individual detection of the metabolites for their quantification or identification, being GC or LC the most used in this area. Then, MS is the commonest detector in metabolomics, both for targeted and untargeted analysis, as it provides a high spectral resolution —and therefore a great accuracy in the measurement of the *m/z* ratio—, or an excellent sensitivity, depending of the given MS approach.

The last two strategies have not been applied for development of the research planned in the present PhD, but training of the student on them has been developed to improve his analytical experience.

1.4. Separation and detection in metabolomics

The high number of metabolites in any vegetal or animal sample makes mandatory implementation of appropriate separation of the sample components before being subjected to detection, mainly in targeted analysis.

Separation techniques used in metabolomics

The most used separation techniques in metabolomics (commonly prior to a high resolution–high sensitivity detector) are GC or LC depending on the nature of the target metabolites. In addition to improved sensitivity and resolution power of the analysis, chromatographic separation provides extra information (retention time) that facilitates identification of the metabolites, especially in dealing with

untargeted metabolomic strategies.

Chromatography is a set of separation techniques where the sample components are distributed between a mobile phase (a liquid in LC, an inert gas in GC, or a supercritical fluid —SF— in the case of supercritical fluid chromatography —SFC) and a stationary phase (the column packing). The separation takes place as a function of the different affinities of the sample constituents towards each phase. GC allows the separation of volatile compounds, such as aromatic compounds, but also compounds that can become volatile after derivatization.

In LC the most determining component in the separation is the column, whose characteristics define the chromatography type and mode. Reverse phase columns, mainly those packed with silica (C18 or C8), are characterized by strong interaction with low polarity compounds, whereas normal phase columns, such as HILIC, are more effective for polar compounds, thus covering a wide range of metabolites with very different features. The combination of both GC and LC provides a higher level of information since there are metabolites that are not volatile and do not yield volatile products, do not ionize adequately at atmospheric pressure or are thermolabile. Therefore, both should be used complementary to get the best coverage of metabolites in the biological sample. However, the majority of metabolites to be separated by GC requires a laborious sample preparation step (hydrolysis, derivatization); thus, its use for clinical diagnostic and large-scale studies can be limited. The increase of new materials that provide new possibilities to LC are replacing GC applications, particularly in the field of high-throughput targeted metabolomics due to the easier sample preparation (no derivatization is not required most times) and the shorter analysis times. However, GC is essential for determination of certain families of metabolites such as fatty acids, carboxylic acids or carbohydrates.

Detection equipment in metabolomics with special emphasis on that used in the present research

Metabolomics analysis demands high sensitivity for detecting metabolites present at low concentrations in biological samples, high-resolution power for identifying the metabolites and wide dynamic range to detect metabolites with variable concentrations in complex matrices. MS spectrometry is the most extensively used analytical technique that fulfils these requirements.

The fundamental of MS is the differential displacement of ionized molecules through vacuum by applying an electrical field. Simplistically, a mass spectrometer consists of a sample inlet, an ion source, a mass analyzer, a detector and a data system. The sample inlet has as function to introduce the sample molecules into the ion source, where they become ionized. The ion source generates gas-phase ions via an ionization technique, the mass analyzer separates the ions according to their mass-to-charge ratio (*m/z*), and the detector generates an electric current from the incident ions that is proportional to their abundances [27].

The required ionization of the target analytes prior to MS detection can be produced by different devices. The most common ionization sources in metabolomics are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Among them, ESI is the most widely used for untargeted metabolomics, mainly because it produces a soft ionization (*viz*, ions are generated with little or no fragmentation, which can help to identify unknown metabolites), and also because it can ionize an extensive polarity range of compounds. This ionization source has been widely applied in clinical metabolomics and it is particularly useful when coupled to LC as ionization occurs at atmospheric pressure [28]. In addition, ESI does not require derivatization of the target analytes, and ionizes a large mass range of compounds from non-volatile to polar compounds.

ESI is a process that creates or transfers intact ions from the solution to the gas phase at atmospheric pressure. In the coupling of an ESI source to LC, the liquid sample from the chromatography column is introduced through the mobile phase into a capillary tube applying a high potential difference (±3–6 kV) between the capillary tube and the counter electrode in positive or negative ionization mode. The electric field causes an accumulation of charge at the liquid surface at the end of the capillary, which break the spray of highly charged droplets. This nebulization is usually assisted by a flow of gaseous nitrogen (the nebulizer gas) flowing through a tube coaxial to the main capillary, aiding in the formation of the aerosol. The drying gas evaporates the solvent in the droplets causing them to shrink. When the repelling forces come close to the cohesion forces, there is a columbic explosion. The droplets undergo series of ruptures resulting in smaller and smaller droplets which will continue to undergo successive evaporation and explosion processes till the repulsive columbic forces are great enough to overcome the forces of the surface tension of the droplet (Rayleigh limit). The fission process continues until each droplet contains only one ion at the end. The evaporation of the remaining solvent leads to formation of single ions with multiple charges (z) to the gas phase, even though metabolomics has interest in z = 1. Once the molecules have been ionized, they are transported to the mass analyzer via an electric or magnetic field.

ESI is a soft ionization technique, the ions produced are mainly derived from the incorporation or transfer of one or more protons to the molecules, although it may also originate ions corresponding to adducts with other species present in the mobile phase. This has been the ionization technique used for development of the research that constitutes this Thesis-Book.

Mass analyzers can be used either alone or combined. The combination can be made between the same type of mass analyzer or between different mass analyzers (hybrid instruments) and is called tandem MS (MS/MS). In this

approach, the ions that arrive at the first mass analyzer (precursor ions) are isolated, subsequently fragmented, and finally the fragment ions are separated according to their *m/z* in a second mass analyzer and detected. For some types of mass analyzers, the number of mass analysis steps can be increased (*i.e.*, the fragment ions can be re-fragmented and further detected. In this case, the experiment is termed multiple-stage MS (MS^n), where *n* refers to the number of mass analysis steps).

Tandem MS and multiple-stage MS improve identification of a given molecule, because not only the molecular ions are detected but also the fragments generated from precursor ions.

The main performance characteristics of a mass analyzer are [29–31]:

(a) Mass accuracy of the measured *m/z* provided by the mass analyzer, directly related to the mass resolving power and stability of the instrument.

(b) Mass resolving power or ability of an MS to provide a specified value of mass resolution (*i.e.*, the instrument generates distinct signals for two ions with a small *m/z* difference).

(c) Mass range or limits of *m/z* within which an MS can detect ions or is operated to record a mass spectrum.

(d) Transmission efficiency or ratio of the number of ions reaching the detector and the number of ions leaving the mass analyzer, related to the sensitivity of the mass spectrometer (*i.e.*, the minimal concentration of a compound leading to a peak intensity greater than a specified signal-to-noise ratio).

(e) Scan speed or rate at which the analyzer measures over a certain mass

range.

(f) Scan cycle time or the time required to obtain a mass spectrum, also known as duty cycle.

MS alone can be used in a metabolomics analysis, being this performance known as direct infusion mass spectrometry (DIMS). Major drawbacks of this approach are ion suppression effects —which make difficult the identification/quantification of metabolites with low concentrations or some metabolite classes that do not ionize as well as others—, and the requirement of a high-resolution mass analyzer, which increases the cost of the analysis.

Mass analyzers to be coupled to LC or GC are similar. However, the different physical state of the eluate from the column requires a different insertion into the detection system. Therefore, whilst in GC the eluate is already a gas, in LC the eluate is a liquid that must be evaporated before being ionized, as explained above in the description of the ESI process.

The particularity that guides the choice of the instrument is the type of metabolomic analysis to be performed, either targeted or untargeted. As the goal of targeted metabolomics is to perform quantitative analysis of specific metabolites (or a defined set of metabolites), the main features for an MS to be used in this metabolomics strategy are transmission efficiency, scan cycle time and scan speed. In this case, single quadrupole (Q), (QqQ), quadrupole ion trap (QIT) and Orbitrap (OT) are the most employed instruments.

The main demanded features for an MS to be used for untargeted metabolomics are mass resolution power, mass range and mass accuracy. In this case, time of flight (TOF), (QTOF), Fourier transform ion cyclotron resonance (FTICR) and OT are the most used.

The methods developed in the experimental part of this Thesis have been based on

a chromatographic separation by LC and subsequent detection by a QqQ instrument such as that described below.

-*The QqQ mass spectrometer* (see Figure 3) consists of an ion source followed by ion optics that transfer the ions to the first quadrupole —a device formed by four parallel rods to which specific direct current and radio frequency voltages are applied [32]. The rods filter out all ions except those of one or more particular *m/z* values as determined by the applied voltage, which is variable, so that ions with other *m/z* values are allowed to pass through.

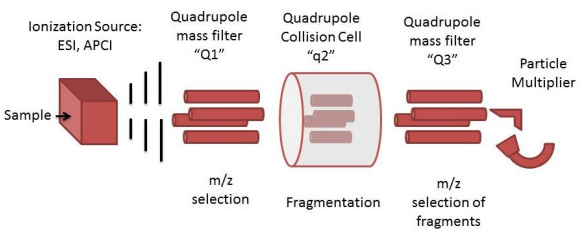


Figure 3. General scheme of a triple quadrupole mass spectrometer.

Afterwards, the selected ions reach a collision cell where they are fragmented. The collision cell —typically known as the second quadrupole— is actually a hexapole filled with an inert gas such as nitrogen or argon. The fragment ions formed in the collision cell are then sent to the third quadrupole for a second filtering step to enable isolation and subsequent examination of multiple precursor-to-product ion transitions. This operational way is called multiple reaction monitoring mode (MRM). Since the fragment ions are pieces of the precursor, they represent portions of the overall structure of the precursor molecule. Due to the low-mass accuracy achieved with respect to other mass analyzers, triple quadrupole

spectrometers are preferably used for targeted analysis, as they allow quantification with high sensitivity and selectivity in the MRM mode, actually the most sensitive operational mode for the triple quadrupole MS instrument.

-*The QTOF mass spectrometer*, together with the TOF instrument are the most used mass analyzers for untargeted metabolomics as they provide data acquisition over a wide mass range with high mass accuracy and resolving power.

Figure 4 shows the diagram of a QTOF mass analyzer [33]. It is based on the same configuration as the QqQ but replacing the last quadrupole by an acceleration tube as mass analyzer (usually an orthogonal configuration) to filter out ions according to the equation of kinetic energy. The QTOF can operate in MS mode with the TOF as scanning tool, by taking benefit from the high mass accuracy, or in MS/MS mode for structural elucidation. This hybrid mass analyzer offers better selectivity than triple quadrupoles meanwhile sensitivity is considerably lower. On the other hand, thanks to its good mass accuracy (below 2 ppm) highly reliable identification can be achieved, thus allowing its use for global metabolic profiling.

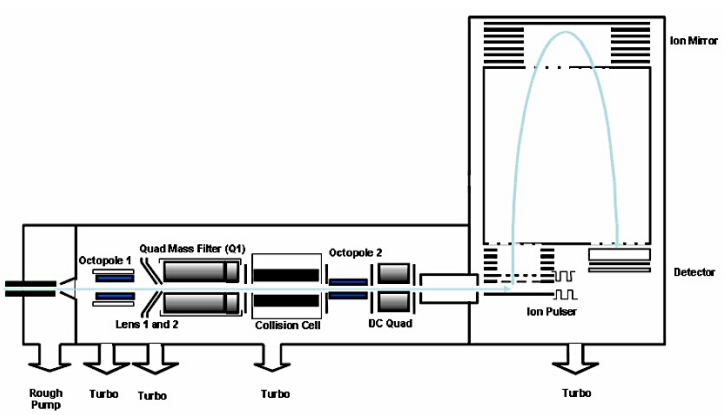


Figure 4. General scheme of a quadrupole time-of-flight mass spectrometer.

SIL-IS and similar approaches used in metabolomics

As previously mentioned, LC–ESI-MS/MS is one of the most frequently used approaches for quantifying endogenous metabolites in biosamples [34]. However, it has to be pointed out that the main drawback of this hyphenated equipment is the ESI interface, which tends to suffer from matrix effects (MEs), either ion suppression or enhancement [35], leading to inaccurate quantifications, especially in dealing with complex matrixes. Ion suppression is caused by the presence of (endogenous or exogenous) coeluting components in the ion source of the MS, which has been attributed to numerous mechanisms including competition for "charges" between analytes and interfering compounds or a change of viscosity and/or surface tension of the droplets in the ion source [36,37].

The two approaches commonly used for accurately quantifying endogenous metabolites are the standard addition method (SAM) and the stable isotope labeled-internal standard (SIL-IS) method [38]. The former works by serial addition of target standards of the analytes into specific sample aliquots to establish the standard addition curve, which is an effective method of calibrating matrix effects caused by quantification errors. The major disadvantage of the SAM is that each sample needs to be analyzed by several runs to obtain the standard addition curve, which makes this approach impractical for clinical analysis where a large batch of samples needs to be analyzed and the amount of sample is limited most times.

The SIL-IS method is currently the gold standard for quantifying metabolites. SIL-ISs are chemically and structurally similar to the target analytes with specific atoms in the analytes replaced by their corresponding isotopes, such as deuterium for hydrogen, ^{13}C for ^{12}C, ^{15}N for ^{14}N, or ^{18}O for ^{16}O. The use of SIL-ISs normalizes the MS intensity of analytes to their isotopic analogues and, therefore, effectively compensates for the matrix effect, ion suppression from other coeluting analytes, and variations caused by sample preparation, injection, and instrument

parameters [39–44]. Because the physicochemical properties of an IL-IS are almost the same as the target analyte, the signal changes caused by MEs can be effectively calibrated by the SIL-IS method [45–47].

Theoretically, since the SIL-IS is almost identical in structure to (and coelutes with) the analyte, the degree of ionization suppression or enhancement caused by the coeluting matrix components should be the same for the SIL-IS and analyte. Therefore, while the absolute response may be affected, the analyte- to-IS peak area ratio should be unaffected and the analytical method should be accurate, precise and rugged. The mass difference between the target analyte and SIL-IS has to be more than 3 mass units to avoid signal contribution. The ^{13}C- or ^{15}N-labeled are often more appropriate as IS than the ^{2}H-labeled ones, because the physical properties of ^{12}C and ^{13}C are more similar as compared to ^{1}H and ^{2}H. However, synthesizing the non-deuterated SIL may be expensive and impractical.

In the absence of SIL standards, structural analogues are used as the second best choice; but their use may result in poor quantification performance, particularly in analyzing metabolites present in a complex matrix [48]. Structural analogues are often not coeluted with the analyte of interest, and therefore, they would experience levels of MEs different from that of the analyte. As a result, the relative signal intensities of a metabolite and its analogue may not reflect their concentration ratio in the sample.

In targeted analyses, the use of internal standards, especially SIL-ISs, is recommended to improve precision and to handle matrix effects; therefore, this has been the approach used in the research in this Thesis-Book. This approach is not a practical tool in broad metabolic profiling approaches in which the number of metabolites is large, they are chemically too diverse to afford a common labeling approach, and many of them may not even be known.

Other tools from which metabolomics takes advantage are standard operating

procedures (SOPs) and quality control (QC) samples. The former could, to some extent, prevent heterogeneity and avoid misinterpretations [49–52]. The use of standard QC and metrics normalizes intra- and interlaboratory metabolomics measurement variations [53,54], reinforced by applying consistent statistical correction methods and appropriate computational tools to overcome some technical variations [55,56].

1.5. Data treatment in metabolomics

Chemometrics can be defined as "the chemical discipline that uses mathematical, statistical, and other tools employing formal logic, to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analyzing chemical data" [57]. This discipline has been fundamental for the development of metabolomics, while growing with it. From design of experiments, through data preprocessing and processing, to data analysis, chemometrics tools are used to design, process, visualize, explore and analyze metabolomics data. Of the different steps involved in data treatment in metabolomics, the following approaches have been used for the data obtained in the research developed in this Thesis.

Data preprocessing

From data acquisition to statistical analysis, metabolomics data need to undergo several preprocessing and pretreatment steps, crucial for data quality and interpretation of the results. The purpose of data preprocessing is the transformation of the raw data (those provided by the analytical instrument that are exclusive of its trademark) into clean data (those that have a universal format) for processing by any software of data processing. In our case it consisted of extraction of the potential metabolites (usually called potential molecular features). The term feature has been defined to be a bounded, two-dimensional LC–MS signal consisting of a chromatographic peak (*i.e.,* retention time) and an MS

signal (*m/z* value).

Data pretreatment

After data preprocessing is complete, data pretreatment consisted of normalization and reducing batch and drift.

- *Normalization*: the main objective of this approach is to remove unwanted variation among samples allowing quantitative comparison of them. In metabolomics, the samples under study are biofluids in most cases, and they exhibit differences in the concentration of metabolites due to variable dilution factors for different samples.

- *Reducing batch and drift effects*: LC–MS instruments are prone to batch and time drift effects owing to changes in instrument sensitivity and intensity, among others. Targeted methods correct batch and drift effects with the inclusion of SIL-IS calculating the ratio between the target compound and the internal standard. This has been the strategy used in the research that constitute this PhD-Book.

Data analysis

After data pretreatment, the data matrix can be analyzed by multivariate or univariate methods to distinguish different diseases or subtypes and defining candidate biomarkers. Metabolomics studies typically generate large amounts of data that complicates the use of univariate statistical analysis. However, if the concentration of a particular metabolite is found to be significantly altered through multivariate analysis, univariate analysis can be used to test the statistical significance of the change. This typically involves the use of the Student's *t*-test or one-way analysis of variance (ANOVA). A more useful statistical approach is multivariate analysis, which can be applied to reduce large volumes of data into a few dimensions for classification and prediction of out-comes. Statistical analysis

strategies employed in metabolomics are designed according to the approach selected, being slightly different for targeted and untargeted analysis.

Targeted metabolomics analysis often requires statistical methods to be used during the development of the optimized analytical methodology for the determination of the compounds of interest. Thus, statistical procedures as response surface or screening design are employed to optimize experimental parameters to obtain the best conditions for determination of the target compounds [58,59]. The rest of statistical analyses employed in the process of developing a new methodology for targeted metabolomics are based on simple approaches such as simple regression, used to obtain the calibration curves for metabolites quantitation, or simple calculations, used to characterize the analytical method (reproducibility and repeatability, mainly).

2.Vitamin D: the target of the PhD research

As the second part of the binomial (metabolomics and vitamin D) that constitutes the edge of the developed research, the main aspects on this vitamin are discussed below.

2.1. Characteristics and metabolism of vitamin D

Vitamin D is a prohormone that comes in many forms, but the two major physiologically relevant ones are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Technically, vitamin D is classified among secosteroids in which one of the rings has been broken (Figure 5). As derived from a steroid, the structure of vitamin D retains its numbering from its parent compound cholesterol: the 9,10 carbon-carbon bond of ring B is broken, as indicated by the inclusion of "9,10-*seco*" in the official nomenclature. The configuration of the double bonds are notated *E* for "entgegen" or *trans*, and *Z* for "zuzammen" or *cis* [60]. Thus, the official name of vitamin D_3, by relation to cholesterol, is 9,10-*seco*

(5Z,7E)-5,7,10(19) cholestatriene-3β-ol; while the official name of vitamin D_2 is 9,10-*seco* (5Z,7E)-5,7,10(19),22-ergostatetraiene-3β-ol. The differences between both are a double bond between C22 and C23, and a methyl group on C24 for vitamin D_2.

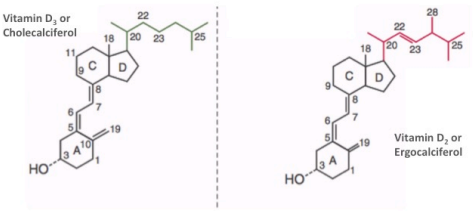


Figure 5. Important forms of vitamin D.

Vitamin D_3, the naturally occurring form, originates from dermal synthesis by conversion of 7-dehydrocholesterol, a precursor of cholesterol, into pre-vitamin D_3 under ultraviolet UV-B radiation, on the plasmatic membrane of skin cells. This process is followed by fast thermal isomerization to vitamin D_3 (Figure 6). Approximately 50% of pre-vitamin D is converted into vitamin D in 2 h, formed in the cell membrane and then transported to the extracellular space. From this, vitamin D is transported in blood bound mainly by its transporter protein DBP to reach the liver. There, vitamin D-25-hydroxylase of cytochrome P450 produces the 25-hydroxylation in the liver to yield 25(OH)D, the main circulating form. D-25-hydroxylase is not strictly regulated in the liver; therefore, an increase of cutaneous production of vitamin D or its intake increase the level of circulating 25(OH)D —both 25(OH)D_3 and 25(OH)D_2. Therefore, the joint measure of both monohydroxylated vitamin D metabolites is used to determine the status of vitamin D.

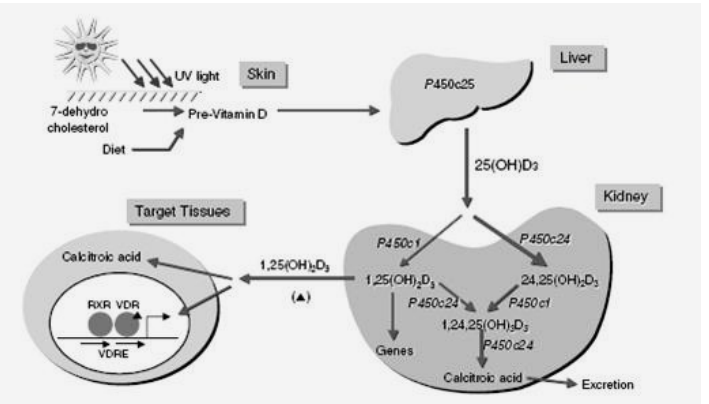


Figure 6. Activation and molecular pathways for vitamin D: integrative schematic synthesis, metabolism and molecular action.

Further 1α-hydroxylation of 25(OH)D both in the kidney and extrarenal sites gives place to the hormonal form, $1,25(OH)_2D$, which mediates its pleiotropic effects through the ubiquitous vitamin D receptor (VDR) that binds to vitamin D response elements in target genes to regulate their transcription [61]. $1,25(OH)_2D$ has potent antiproliferative and cell differentiation-inducing activities, in addition to its role in calcium homeostasis [62]. The inactivation of vitamin D is carried out by side chain oxidation by the mitochondrial 24-hydroxylase, which catalyzes the conversion of both $25(OH)D_3$ and $1,25(OH)_2D_3$ into a series of 24- and 23-hydroxylated products targeted for excretion, culminating in the water-soluble biliary metabolite 26,23-lactone and calcitroic acid [63]. C3-epimerization is a second biochemical pathway via which the major vitamin D metabolites [$25(OH)D_2$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$] are converted to their respective epimeric forms and are further metabolized through the C-24 oxidation pathway [64].

Even though $1,25(OH)_2D$ is in fact the biologically active form of vitamin D, serum $1,25(OH)_2D$ provides no information about vitamin D status and it is often normal or even elevated due to secondary hyperparathyroidism associated with vitamin D deficiency. On the contrary, $24,25(OH)_2D_3$ is the major circulating dihydroxylated vitamin D metabolite in human serum with concentrations between 0.7–40 nmol/L [65,66]. Serum $24,25(OH)_2D_3$ concentrations show a strong positive correlation with serum $25(OH)D_3$ levels, which are approximately 10% of $25(OH)D_3$ levels [66–68].

2.2. Clinical aspects of vitamin D

This section involves aspects such as the clinical samples used for vitamin D determination, the stability of the most frequently used samples, half-life and normal levels in humans of vitamin D and its metabolites, main sources of vitamin D and supplements on this vitamin, as well as its role in human health.

Clinical samples for determination of vitamin D

Biological matrices for determination of vitamin D and its metabolites in humans include dried blood spots (DBS) [69–71], urine [72], saliva [73], cerebrospinal fluid [74], but mainly blood.

DBS collection is a minimally invasive sampling to obtain blood samples on cards of filter paper. Therefore, it is a practical way to screen for vitamin D deficiencies in large epidemiological studies or in at risk populations, where venipuncture is impractical, including pediatric populations, forensics research, rural clinics, and developing countries, or where blood volume is limited as in neonates [75]. Calibration of DBS analysis is difficult owing to the absence of blank samples in the case of endogenous analytes, and the fact that current reference analyses are performed using plasma or serum samples and not dried blood [71].

Saliva as sample for measurement of vitamin D is interesting from the perspective

that it is likely to reflect the free, non-protein-bound hormone in plasma [76], and may serve as a better indicator of vitamin D status [77]. Concentrations of salivary $25(OH)D_3$, however, are at the picomolar ranges, less than one-thousandth of that in plasma, and require sensitive assay technology for measurement [78]. A good correlation was found between the serum and salivary $25(OH)D_3$ levels [73]

Urine is a more complicated sample for vitamin D analysis as most of its metabolites are conjugated; therefore, in addition to volume normalization, if required, sample preparation involves hydrolysis (usually catalyzed by β-glucuronidase, followed sometimes by derivatization). Ogawa *et al.* found the amount of $24,25(OH)_2D_3$ in urine 2–3 times greater than that of $25(OH)D_3$, contrary to the 1:10 ratio of $24,25(OH)_2D_3$ to $25(OH)D_3$ in serum [72]. Sulfate conjugated vitamin D metabolite $25(OH)D_3$ 3-sulfate has also been found in human plasma at levels similar to or higher than that of $25(OH)D_3$ in adults or infants, respectively, but lower in preterm infants [79]. This conjugate has not been proven to have significant biological activity [80].

Cerebrospinal fluid from multiple sclerosis patients was used to study the controversial information on the effect of vitamin D metabolites on the disease. The concentration of 25(OH)D in this biofluid from the target patients was not significantly different as compared with patients with other inflammatory neurological diseases, but the cerebrospinal fluid/serum ratio was significantly lower in multiple sclerosis patients [74]. The authors of this study stated that the results they obtained do not support that 25(OH)D is actively transported to the cerebrospinal fluid, or that the cerebrospinal fluid or serum levels or their ratio exert a major impact on multiple sclerosis activity. A comprehensive metabolomics study on the role of vitamin D metabolites in multiple sclerosis patients has not been developed so far [81].

Blood, either as serum or plasma, is with a high difference the most used biofluid for clinical studies of vitamin D and its metabolites in such a way that the reference

values for the status of vitamin D are established in one of them.

Plasma or serum have been the clinical samples preferred by the research team in which the PhD student is integrated, for development of methods for determination of vitamin D and its metabolites. The team has worked on the metabolism of vitamin D since 1997, when metabolomics had not been defined as such omics discipline. Seven articles were published by the team between 1997–1999 dealing with methods to improve the determination of vitamin D and metabolites using plasma as clinical sample [82–88]. Key improvements in sample preparation [82–85] and in detection using a conventional UV-visible detector [86], a conventional fluorimetric detector [87] or a laser-induced fluorescence detector [88] (in all instances after LC separation) were developed during the three-year interval. A review on the state-of-the-art and trends on the determination of vitamin D and its metabolites was also published in 1999 [89] that, together with the research [82–88] completed the PhD of one of the team's students.

Further studies on vitamin D metabolites developed by the research team (between 2007 and 2013) were based on serum as clinical sample, and mainly devoted to reduce the sample size, automation of sample preparation, improvement of sensitivity and cross-sectional studies [90–98].

As no comparative studies were found in the literature on the best sample from blood for the analysis of vitamin D_3 and metabolites involving, $1,25(OH)_2D_3$ in addition to $24,25(OH)_2D_3$ and $25(OH)D_3$, a study of the PhD student was focused on comparison of both types of sample. With this aim four different Vacutainer™ tubes were used: plastic serum tubes with spray-coated silica (serum), plastic serum tubes with spray-coated silica, and a polymer gel to favor serum separation (serum-gel), spray-coated silica tubes with heparin for plasma (plasma) and heparin tubes with polymer gel to favor plasma separation (plasma-gel). The physical barrier formed by the gel between serum or plasma and blood cells during centrifugation allowed setting a more efficient separation as compared to conventional tubes. The results showed

that serum and plasma reported high accuracy (above 83.3%) for vitamin D and metabolites, while precision, expressed as relative standard deviation, was below 12.9% for all analytes in both samples. Statistical analysis revealed that serum and plasma provided similar physiological levels for vitamin D_3, 24,25(OH)$_2D_3$ and 25(OH)D_3, while significantly different levels were obtained for 1,25(OH)$_2D_3$, always higher in plasma than in serum [99]. The fact that plasma was not selected for subsequent studies was owing to the higher complexity of this sample as compared with serum that deteriorates faster and gives place to dirty chromatograms, where the small peaks from low-concentrated metabolites are hardly distinguishable from the noise.

Stability of serum/plasma samples for analysis of vitamin D

Vitamin D and its metabolites have traditionally been considered unstable compounds sensitive to light and temperature [100]; therefore, studies on their stability have been developed, but not in depth. A study published in 2004 was devoted only to the 25(OH)D metabolite, which was determined by a chemiluminescence immunoassay method in serum and plasma samples both fresh and frozen [101]. Comparison of the results showed *p* values greater than 0.7, indicating no significant difference between fresh and frozen samples, the latter subjected to five freeze–thaw cycles. Thus, it seemed to be demonstrated that the samples did not need to be frozen if they are assayed within 5 days since sampling. The equivalent cross-reactivity of 25(OH)D_2 and 25(OH)D_3 in the target immunoassay used was also demonstrated. Cross-reactivity to the dihydroxyvitamin D metabolites was accepted, but without clinical concern due to the approximately 1000-fold concentration difference between these metabolites and the 25(OH)D in the circulation. As the authors stated, concentrations of 1,25(OH)$_2$D at five times normal values would contribute less than 0.05 nmol/L to the measured 25(OH)D concentration. Thus, pathological 1,25(OH)$_2$D concentrations would be expected to contribute less than 0.1% to the overall

imprecision. A further study in serum also involving only the 25(OH)D metabolite but with radioimmunoassay (RIA) analysis showed stability of this compound at –25 °C in storage between 6 and 24 years [102].

In a more recent study on stability of the 25(OH)D metabolite in serum, the levels determined by LC–MS/MS were compared with those obtained by conventional RIA, with no distinction between $25(OH)D_3$ and $25(OH)D_2$ [103]. Note that $1,25(OH)_2D_3$, less concentrated, was not determined. The values obtained by the RIA method exhibited a mean bias of about 8.35 ng/mL, most likely as a result of cross-reaction of the antibody with low-abundant metabolites, including $24,25(OH)_2D_3$. Various preanalytical factors, such as long sample sitting prior to serum separation, repeated freeze–thaw cycles, and the presence of anticoagulants had no significant effects on the determinations.

After checking the scant number of vitamin D metabolites involved in previous stability studies, the interest of the PhD student was focused on knowing how the different vitamin D metabolites behave under different storage conditions and situations encountered in the analytical laboratory. Thus, the influence on the target analytes of temperature storage (room temperature, refrigeration at 4 °C, frozen at –20 °C or at –80 °C), lyophilization, and the number of freeze/thaw cycles of serum samples were studied for a two-month period [104]. The different effect of the storage temperature on the analytes stability, the limitation of the freeze/thaw cycles to only two owing to their destabilization effect, and the stability conferred by lyophilization were the most remarkable results of the study.

Half-life and levels of vitamin D metabolites in humans

The parent sterol vitamin D has a half-life close to 24 h [105], relatively short as compared with 25(OH)D with a half-life of 2–3 weeks [106]. Therefore, 25(OH)D measurement is a better indicator of vitamin D storage, whether obtained from sunlight (UV exposure) or dietary sources. The most potent physiologically active

circulating metabolite produced by humans is 1,25$(OH)_2$D, which has a half-life of 4–8 h [107]. While 25(OH)D circulates at the nmol/L concentrations, 1,25$(OH)_2$D is present at the pmol/L concentrations, at a 1000-fold lower concentration than 25(OH)D (with the reference interval for healthy adults being 38–134 pmol/L [108]), which means that this dihydroxymetabolite represents the greater challenge in assay development.

The levels of vitamin D metabolites that defines normal, abnormal or pathological values in adult humans are established with respect to the circulating form. There is virtually unanimous agreement that a serum level of 25(OH)D less than 30 nmol/L defines vitamin D deficiency, but there are opinion differences regarding the 25(OH)D levels that define vitamin D insufficiency which may differ from 50 nmol/L [109] to 75 nmol/L [110]. The Institute of Medicine recommends that deficiency corresponds to <30 nmol/L, and places a person risk relative to bone health; 30–50 nmol/L places some, but not all, persons at risk for inadequacy, which can be considered as insufficiency; while sufficiency (adequate) is established for ≥50 nmol/L that meets the needs of 97.5% of the population. About 1 billion people worldwide are estimated to have 25(OH)D levels of less than 75 nmol/L [111]. The Institute of Medicine also states that levels higher than 75 nmol/L are not consistently associated with increased benefit [112]. Table 1 summarizes the levels of both vitamin D and 25(OH)D as expressed by K. Poongkodi [113] including extreme values.

It has also been proposed that free or bio-available 25(OH)D provides a better assessment of vitamin D sufficiency than total 25(OH)D [77]. Free or bio-available 25(OH)D can be calculated taking measured vitamin D binding protein (DBP) and albumin into account [114] or can be directly measured by immunoassay [115]. The 24,25$(OH)_2D_3$ to 25$(OH)D_3$ ratio may also be a predictor of serum 25$(OH)D_3$ response to vitamin D_3 supplementation [68,116].

Table 1. Levels of Vitamin D and 25(OH)D in human serum.

Status	25(OH)D (ng/mL)	Vitamin D (nmol/L)
Severe deficiency	<10	<25
Deficiency	<20	<50
Insufficiency	21 – 29	50 – 74
Sufficiency	30 – 100	75 – 250
Optimal	30 – 60	75 – 150
Toxic	>150	>375

Differentiation between the concentration of 25(OH)D and its epimer 3-epi-25(OH)D in infants is of interest in this population as studies in up to 1 year of age have shown significant 3-epi-25(OH)D concentrations as high as 200 nmol/L with relative contribution to total 25(OH)D as high as 55% [117–120]. This behavior can be attributed to either transference of the epimer in the utero or to postnatal formation.

Sources of and supplementation with vitamin D

Vitamin D is derived from two major sources in humans, with approximately 80–90% produced on the skin resulting in cholecalciferol (D_3), and the other 20% derived from dietary sources, which can be animal cholecalciferol (D_3) or plant-derived ergocalciferol (D_2). The latter originates from the yeast and plant sterol ergosterol, and is obtained from diet or supplements.

Small amounts of vitamin D can also be obtained by nutritional intake of either vitamin D_3 or by foods fortified with vitamin D_2. Both forms undergo a substrate-dependent liver hydroxylation to 25-hydroxyvitamin D [25(OH)D_2 and 25(OH)D_3], as stated in Figure 6.

The perceived conception that vitamin D supplementation can prevent, improve or

cure chronic disorders has caused over the last years a massive rise in demand for measurement of 25(OH)D in blood as surrogate marker of vitamin D status.

There is much interest in intermittent dosing for patient convenience and long term adherence, but caution is warranted when using supraphysiological bolus doses as these may be counterproductive leading to transient vitamin D intoxication in the immediate post-dosing period [121–123] or adverse effects in the longer term [124,125]. There are not conclusive results on vitamin D supplementation. Thus, interventional studies on vitamin D supplementation (1.200 IU vitamin D per day) in patients with Parkinson disease showed no conclusive results when compared with individuals who received placebo during the same period [125]. On the contrary, 5 studies of vitamin D supplementation in rodents on the effect of vitamin D therapy on substantia nigra dopamine neurons resulted in: one study with a significantly higher dopamine level and two a significantly higher concentration of the enzyme promoting conversion to dopamine (tyrosine hydroxylase), in the substantia nigra of the vitamin D supplemented rodent group [126,127]. One of these studies also demonstrated that oxidative injury of the substantia nigra was significantly lower after vitamin D supplementation [126]. This result was in line with another study reporting higher neuronal survival in the substantia nigra when $1,25(OH)_2D$ was injected [128]. However, very high concentrations of $1,25(OH)_2D$ enhanced neurotoxity, as also found in Klotho-insufficient mice that resembled human aging and exhibit abnormal levels of serum $1,25(OH)_2D$ caused by abnormally high vitamin D metabolism in the kidney [128,129].

In infant studies, vitamin D supplementation (2.000 IU/day) in the first year of life increased the risk of developing atopy, allergic rhinitis, and asthma late in life [130].

The role of vitamin D and its metabolites in human health

Over the past decade, more than 1600 studies have been conducted on vitamin D, and more than half of them are cohort or observational studies demonstrating an association between deficits in vitamin D and a litany of acute and chronic disorders (cardiovascular disease, cancer, diabetes, fractures, depression and respiratory tract infections, to name a few) [131]. These findings have fueled the hypothesis that vitamin D supplementation —a widely available, low-cost and mostly harmless intervention— might treat or even prevent these disorders. Association, however, is not causation.

At present, evidence supports vitamin D supplementation to help prevent fractures (particularly if given with calcium), and possibly to prevent falls and slightly reduce mortality (particularly in older patients, >70 years of age). No other effects have been proven. For many other conditions, the evidence for vitamin D supplementation is plagued by the use of small, poor-quality trials. Lastly, testing of 25(OH)D levels in the general population is not necessary, and very high doses should be avoided.

Clinical interest in Vitamin D and its purported roles not only in calcium and bone metabolism but in several other medical conditions (diabetes, cardiovascular disease, multiple sclerosis, cancer, psychiatric disorders, neuro-muscular disease) has led to a surge in laboratory requests for 25(OH)D and $1,25(OH)_2D$ measurement, but also their ratio and ratios of other vitamin D metabolites. The search for vitamin D metabolites-based biomarkers that could be associated to given diseases is other of the open fields for interesting research.

Contributions of the research team to ensure a proper use of the metabolomics in the field of vitamin D metabolites as diseases biomarkers started with the publication of a review on the analytical process to search for metabolomics biomarkers, to establish the basis for use of the proper analytical step in each case [132]. Other

contributions have constituted calls of attention on the unclarified action of the different metabolites of vitamin D on multiple sclerosis and the role of metabolomics to clarify the vitamin D–multiple sclerosis relationship [81,133].

The research of the PhD student also constitutes a contribution to the establishment of the normal levels of vitamin D as the data from the analyses of massive numbers of serum samples from different cohorts have provided interesting results as discussed in the publications on them [134–137].

2.3. General analytical aspects of vitamin D and its metabolites

Sample preparation

The critical step of sample preparation for determination of vitamin D in the clinical field has been the workhorse of most analytical chemists working in this field, who have looked for interferents removal, automation and preconcentration steps to decrease sample volume as much as possible, increase sensitivity, selectivity and the number of analytes to be determined, and shorten the analysis time. Therefore, traditional sample preparation steps have consisted of:

Deproteination or removal of protein and related macromolecules, a common step with serum or plasma samples. The step involves mixing equal volumes of sample and precipitant reagent such as acetonitrile (ACN) [138–141], ammonium sulfate [142,143] or ethanol [144,145]. This is the only preparation step prior to selective quantitation (*e.g.*, to RIA).

Saponification, mainly used with samples with high content of lipids such as infant formulae [145,146], enriched milk [148–150], eggs [151], fish oil [151–153] or margarine [154]. This step substitutes deproteination and is followed by (LLE). Saponification and subsequent removal of lipids involve losses of vitamin D and its metabolites by dragging; losses that have been traditionally evaluated by a recovery study using radioactive isotopes.

LLE constitutes an alternative to the two previous sample preparation steps. It can be simple or multiple LLE. The most general manual procedure was that of Bligh and Dyer [155].

SPE, used since the earliest methods for quantitation of vitamin D and its metabolites, underwent a remarkable expansion with commercialization of SPE cartridges, which virtually substituted manual column packing. The improvement of sample preparation achieved by SPE can be summarized in: lower amount of neutral lipids in the extract as compared with LLE; higher protection of the equipment used for subsequent individual separation (LC or GC) or for direct quantitation by competitive protein binding (CPBA) radioreceptor binding (RRB) or RIA; effective decrease in costs in terms of cartridges and solvents; high preconcentration factors by final elution with small eluent volumes; and, availability for selective separation of the analytes based on polarity differences. Nevertheless, SPE also involves some drawbacks such as: necessity for calibration procedures to minimize the variability among commercial cartridges; potential introduction of contaminants that can remain in the sorbent and might be eluted in subsequent steps; excess of confidence in the cleanup capacity of the procedures, with absence of checking and control steps.

Liquid chromatography preparation as step previous to individual chromatographic separation. Molecular exclusion [156,157], solid–liquid partitioning either by normal [158–160] or reverse-phase chromatography [156,161,162] or a combination of them [157,161,162] have been used prior to RIA [162] or CPBA [163].

An overview of the contributions to sample preparation for determination of vitamin D and its metabolites by the PhD-student research team and by him himself are as follows:

A first contact of the team by the last decade of the XX century with its new research

line was to improve a previous sample preparation SPE procedure for plasma found in the literature [164]. Keeping protein precipitation by ACN the SPE step was improved by more efficient mixtures used for washing and elution from two subsequent cartridges (Bond-Elut C18 and Bond-Elut silica) [82]. A drastic decrease of interferents (a cleaner LC–UV-chromatogram) allowed the determination of 24,25(OH)$_2$D, 1,25(OH)$_2$D, in addition to 25(OH)D, the only determined in the previous procedure [164].

An on-line coupling of a flow-injection (FI) manifold to the loop of the injection valve of an LC–UV detector arrangement allowed partial automation of the SPE step by locating a C18 minicolumn in the loop of the FI injection valve and selecting sequential passage through it of the conditioning solution, the sample, washing solution, and eluant [83]. The continuous method provided as main advantages a single and miniaturized SPE (smaller amount of sorbent), reusability of the minicolumn, drastic preconcentration factor and simple automation. The method thus developed was improved by a more effective protein precipitation by changing ACN to isopropanol, then compared advantageously with an RIA method for 25(OH)D [84]. The use of an aminopropyl-silica sorbent and the same continuous arrangement showed the following advantages provided by this polar sorbent instead of the nonpolar C18 sorbent [85]: lower detection and quantitation limits and better CV % values and recoveries than the previous methods. A new application of the arrangement allowed the determination of vitamins D_2, D_3, K_1 and K_3, and also the 25(OH)D_3, 24,25(OH)$_2D_3$ and 1,25(OH)$_2D_3$ metabolites [86]. Other improvements involved an LLE step prior to SPE and the same continuous system, including postcolumn derivatization and changing the UV-chromatographic detector to a conventional fluorimetric detector (sensitivity increased about 50 times with respect to previous methods) [87] or a laser-induced fluorescence detector (sensitivity 10 times higher than with the conventional fluorimetric detector) [88].

Almost all subsequent developments of the team in this research line during the XXI

century involved commercial automated high-pressure SPE stations (Prospekt1, Prospekt2 or Symbiosis Pharma, all from Spark-Holland) on-line connected to the chromatograph in such a way that the mobile phase acts as eluent, thus inserting in the chromatographic column the total amount of the compounds retained in the CN cartridge. The first contribution —for determination of 25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$— involved a first manual LLE step followed by the SPE step by Prospekt1 and the final LC–DAD (diode array detector) step. A cleaner chromatogram, better reproducibility and shorter analysis time were thus achieved [90]. A subsequent development allowed total automation of the method by the use of a robotic workstation to develop the LLE step. In this way, vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites were determined (the wavelength of maximum absorption for each analyte was selected taking advantage of the DAD) [91]. A further application allowed vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites to be determined in the normality range of these compounds in healthy individuals within the 18–80 year-old interval [92]. Avoidance of the LLE step and use of the serum sample mixed with 0.5 mL of ACN containing sodium dodecyl sulfate allowed direct insertion in the Prospekt1 system and chromatographic separation–DAD determination of fat soluble vitamins A, D_2, D_3, α- and δ-tocopherol, and 25(OH)D_3 in 20 min [93].

A key improvement of the methods for the target compounds was achieved by change the DAD to a triple quad mass spectrometry (QqQ-MS) detector. The first application of this detector, always coupled to LC equipment, involved manual sample preparation consisting of protein precipitation with methanol, LLE with hexane, evaporation of the extractant and reconstitution of the residue with methanol prior to injection in the chromatograph for separation of vitamins A, K_1, K_2, D_2, D_3 and E (as α- and δ-tocopherol), and the 25(OH)D_2, 25(OH)D_3, and 1,25$(OH)_2D_3$ metabolites [94]. The use of the commercial SPE station Prospekt2 on-line connected to the LC–QqQ-MS arrangement allowed to work with only 0.2 mL of serum sample injected (after protein precipitation) into the sample loop of the SPE station provided with

Hysphere cartridges automatically conditioned. The analysis time was 20 min [95]. The features of the method made feasible its application to cross-sectional studies [97,98].

One of the innovations in the equipment for sample preparation used by the PhD-student was the present SPE workstation also from Spark-Holland: the Symbiosis Pharma model that substituted Prospekt2. The former was designed for high sampling throughput for which an autosampler for up to 90 samples can be stored at 4 °C. Improvements in the cartridge sorbents and in depth optimization of volumes and times for the equilibration, sorption and elution steps were achievements of the PhD student (see Chapters 1–4).

Derivatization

Derivatization steps based on dehydration [165–168], cycloaddition [169–172], silanization [173,174], or charge-transfer complex formation [175] have been implemented as a pre- or postcolumn step in either LC or GC.

Dehydration reactions occur by exposure of vitamin D and its metabolites to high temperature. The non-specific reaction produces B-ring cyclation to yield pyro and isopyro isomers. The number of isomeric structures thus produced significantly complicates subsequent individual separation and identification. Cyclation constitutes a hard limitation for development of methods involving GC. Selective cyclation to obtain given products [164] or more thermostable products by isotachysterol formation has been reported prior to GC–MS [164].

Diels-Alder cycloaddition contributes to enhancing sensitivity and selectivity in the determination of vitamin D and its metabolites, a reaction that can be developed by very different reagents and has been implemented both in pre- and postcolumn location in LC–MS methods [161,170,172].

Silanization reactions have been used to minimize unspecific adsorption of vitamin

D and its metabolites in GC, mainly owing to the presence of hydroxyl groups on these molecules. A number of silane-derivatives have been used with this aim [138–143, 176–178].

Charge transfer-complex formation has also been implemented, mainly for the determination of vitamin D in pharmaceuticals [70].

Derivatization reactions have been applied by the research team, always in poscolumn location, with the aim of increasing sensitivity as the necessary selectivity was supplied by the LC separation. A Diels-Alder reaction using 4-phenyl-1,2,4-triazolie-3,5-dione (PTAD) formed an adduct with maximum absorption at 337 nm, which increased the sensitivity 5-times with respect to the method without derivatization [86]. A dehydration method favored by a strong-acid medium was also postcolumn developed which improved fluorimetric detection (with conventional [87] or laser-induced [88] detectors).

Sample volume

The sample volume of either serum or plasma required for determination of vitamin D metabolites has experienced a spectacular decrease thanks to the improvement of sample preparation steps, and increased sensitivity of the detectors used (mainly MS detectors). Thus, the isotope dilution-mass fragmentography assay for $1,25(OH)_2D$ published in 1979 [179] required 20 mL of serum subjected to LLE with a chloroform–methanol mixture after addition of [26-$^2H_3$]-$1,25(OH)_2D_3$, and purification by LC. Then, the metabolite in the purified material was converted into the trimethylsilyl derivative and analyzed by GC–MS. The lower limit of quantification (LLOQ) was 13 pmol/L (5 pg/mL), with a CV of 5%; but the large sample volume limited the general applicability of the assay. The evolution of analytical equipment and sample preparation devices led to a method published in 2010, based on LC–MS/MS [180] by which both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ could be measured in 30 µL of sample. An LLOQ for $1,25(OH)_2D_3$ of 15

ng/L (36 pmol/L), a CV of 5–15 % across physiological concentrations, and a total run time per sample of 30 min supported the excellent features of the method. Sample preparation involved a complex on-line process using a perfusion column, followed by a chain of two monolithic columns to clean and enrich the sample prior to LC–MS/MS analysis without derivatization. A recently published method uses a 20 μL serum sample volume for determination of four vitamin D metabolites [*viz.* 25(OH)D_3, 3-epi-25(OH)D_3, 25(OH)D_2, and 24,25$(OH)_2D_3$; with LOQ of 0.091, 0.020, 0.013, and 0.024 ng/mL, respectively]. The very long sample preparation process involved SPE on wells plates, incubation, evaporation, and derivatization for 1 h prior to injection into the LC–MS/MS system [103].

In the contributions from the research team the maximum sample volume used was 2 mL [82–88,90,91], then reduced to 1 mL after exhaustive optimization [92,94,96], and to 0.5 mL by eliminating the LLE step [93]. Finally, 0.2 mL was the volume required when the Prospekt2 workstation was used.

In the research developed by the PhD student the sample volume has been 240 μL in all instances as a compromise between the required sensitivity for determination of the more diluted metabolites and the possibility of obtaining samples from patients in critical state [99,181,182].

Analysis time

A sometimes crucial analytical parameter as analysis time is very difficult to be deduced from most of the publications on this subject. A number of published methods for determination of vitamin D and its metabolites only specify the time required for the chromatographic–detection steps, despite obtainment of the analytical sample [183] has involved deproteinization, centrifugation, LLE, evaporation of the extractant to dryness one or several times, derivatization with enough completion requiring 1 h or even more, etc.

In the research developed by the PhD student, once the method for determination of vitamin D and its metabolites was enough validated for massive application, the time required for analysis is 15 min, achieved by overlapping the chromatographic–detection steps of one sample with the SPE of the next [99,181,182]. The only treatment after sample thawing is centrifugation/filtration prior to location in the thermostated autosampler.

2.4. Methods for determination of vitamin D and its metabolites

Methods for vitamin D metabolite measurement can be divided into two main groups: immunochemical methods and those based on liquid chromatography separation, mainly with subsequent MS detection.

Immunoassay methods

Immunoassays dominate 25(OH)D testing (90%) in clinical laboratories and have developed from laborious manual RIA formats to fully automated assays on random access analyzer platforms. These automated immunoassays are mainly based on competitive principles (Ab or protein binding formats) but recently, a promising sandwich assay based on the metatype antibody principle has become available [184,185]. Automated immunoassays seem attractive for reasons of high throughput capabilities. However, they have some major shortcomings such as limited sensitivity and dynamic range, difficulties in DBP displacement, non equimolar detection of 25(OH)D_2 and 25(OH)D_3, interferences from heterophilic antibodies, and from gel and clot activator in blood collection tubes [186], lack of adequate standardization, and cross-reactivity towards other major circulating vitamin D metabolites, 24,25$(OH)_2D_3$ being the most abundant. Particularly, in specific populations with altered DBP status, as in dialysis patients, intensive care unit patients and in pregnancy, immunoassays show remarkable differences when compared to LC–MS/MS which have been attributed to inefficient release of 25(OH)D from DBP [187–189].

Immunoassay manufacturers correct for $24,25(OH)_2D_3$ cross reactivity in a fixed manner, which may lead to overcorrection, and to falsely low 25(OH)D as a consequence result, in patients with lowered $24,25(OH)_2D_3$ levels, as in chronic kidney disease [190,191].

The most commonly used methods for $1,25(OH)_2D_3$ quantification are competitive RIA with ^{125}I as the radio-label, or enzyme immunoassays that require extensive sample purification to minimize contribution of other vitamin D metabolites [192]. In vitamin D intoxication, elevated levels of 25(OH)D can interfere with $1,25(OH)_2D_3$ measurement using RIA owing to 25(OH)D cross-reactivity of the $1,25(OH)_2D$ antibody [193–195]. More recently, fully automated chemiluminescence immunoassays, which accurately and precisely measure $1,25(OH)_2D$ as shown by good correlation with well validated LC–MS/MS assays, have become available [196–201].

An area of concern in relation to immunoassays is the variability in the detection of $25(OH)D_2$. Some assays claim to have 100% cross-reactivity with exogenously added $25(OH)D_2$ and $25(OH)D_3$ and are therefore equipotent for the measurement of the two metabolites. Other assay manufacturers admit to lower cross-reactivity with exogenous $25(OH)D_2$ (75% [kit insert from IDS, Boldon, UK], 52% [product insert from Abbott, North Chicago, IL]), while some assays were specifically designed to measure only $25(OH)D_3$ (product insert from Roche, Indianapolis, IN). Reports have confirmed the variability of commercial immunoassays to detect $25(OH)D_2$ [202–205].

Immunoassay remains the predominant mode of measurement for 25(OH)D, although problems with equimolar recovery of the D_2 and D_3 metabolites remain an issue.

Almost all immunoassays show a high cross-reactivity with $24,25(OH)_2D$, which increases in concentration with increasing sun exposure; and as 25(OH)D

increases and/or is metabolized to 1,25$(OH)_2$D, which provides an increased supply of the two substrates for the 24-hydroxylase enzyme. Concentrations in the range of 10–15 nmol/L have been recorded for 24,25$(OH)_2$D in serum using GC–MS [206], with reported circulation levels of 10–15% of those reported for 25(OH)D.

Concerns have been raised about a possible contribution to 1,25$(OH)_2$D measurement from other 1α-hydroxylated metabolites [207], and cross-reactivity for 1,25$(OH)_2D_3$ 26,23-lactone, 1,24,25$(OH)_3D_3$, and 1,25,26$(OH)_3D_3$ has been demonstrated in both the Diasorin and IDS assays [208].

There have been major advances in semiautomation and full automation of immunoassays utilizing nonradioactive tracers which have been incorporated into specialist-dedicated immunoassay systems.

Methods involving chromatographic separation

Liquid chromatography–tandem MS (LC–MS/MS) is regarded as the gold standard method for measurement of serum 25(OH)D concentrations due to its inherent analytical sensitivity and specificity [209–213], but the approach is equally powerful to detect other relevant vitamin D metabolites as well, either in single or multiple analyte format. The simultaneous measurement of vitamin D metabolites may provide better understanding of vitamin D metabolism in health and disease and predict which metabolite, or even combination of metabolites may be the best indicators of vitamin D status.

Chromatography-based methods for vitamin D metabolite analysis are less susceptible to matrix effects than immunoassays. LC separations with subsequent detection either by molecular absorption or emission detection have now largely been replaced by LC–MS/MS. This last approach has the advantage of selectivity, accuracy, and precision in analytical measurements as well as multiplexing

capabilities, features that often greatly exceed those of immunoassays. LC–MS/MS is an approach that requires expensive hardware and technical expertise operating in a batch-wise mode with limited throughput, features that may explain why only 10% of clinical laboratories use LC–MS/MS for vitamin D measurement. Accurate and sensitive measurement requires optimization of many steps including sample preparation, calibration, mode of chromatographic separation, choice of internal standard, mode of ionization and transition selection for mass spectrometric detection [214]. Among the various ionization modes, ESI and APCI are the most commonly used in MS vitamin D analysis, with minor use of atmospheric pressure ionization. There are reportedly over 50 vitamin D metabolites [215], apart from numerous chemically synthesized analogs [216] and these must also be considered potential sources of assay interference. Most of the existing LC–MS/MS methods have adequate sensitivity for the measurement of $25(OH)D_3$, $25(OH)D_2$ and their respective C3-epimers, but the improvement of sensitivity required to include measurement of other relevant vitamin D metabolites that circulate at much lower concentrations is not always possible.

This is the case with quantification of $1,25(OH)_2D$, as it is present in serum at extremely low concentrations. The co-existence of many other more abundant vitamin D metabolites that can interfere in $1,25(OH)_2D$ measurement constitutes an additional drawback. The LC–MS/MS platform has allowed development of methods of choice for $1,25(OH)_2D_3$ analysis as it generates a higher selectivity in measurement when compared to immunoassays. Still, the accurate quantification of $1,25(OH)_2D_3$ by LC–MS/MS is a challenge because of its low serum concentrations and lack of ionizable polar groups that result in poor ionization efficiency in ESI and APCI. Furthermore, specific care is needed to avoid potential interference from other dihydroxylated vitamin D metabolites, such as (3-epi) $24,25(OH)_2D_3$, $23,25(OH)_2D_3$, $25,26(OH)_2D_3$ and $4\beta,25(OH)_2D_3$, as they have the same molecular masses and fragmentation patterns. To enhance the detection response of the poorly ionizable compounds, derivatization strategies have been

employed, mostly using Cookson-type triazoline-diones to react with the diene moiety of vitamin D, which enhances stability across the diene, and lends polar side chains to enhance ionization [217–221]. In some cases, mobile phase additives for adduct formation, such as ammonium, lithium or methylamine have been used to further improve ionization efficiency [180,221,222]. Recently, a new, commercially available reagent [Ampliflex™ Diene (AD), AB Sciex, USA] was developed for derivatizing $1,25(OH)_2D$, which results in a 10-fold higher signal-to-noise ratio compared to PTAD [223]. The reaction product is optimized for MS/MS analysis due to its quaternary amine functional group and activated dienophile moiety. In contrast to PTAD, the fragmentation of the AD-$1,25(OH)_2D_3$ product is limited to several defined peaks with the quantifying product containing the entire $1,25(OH)_2D$ structure, which results in different *m/z* values for the $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ product ions. This is beneficial in preventing isobaric cross talk between the two analytes. Also, the AD-$1,25(OH)_2D$ metabolite products are more polar, but remain soluble in organic solvents. This hydrophilic property of the derivatization reaction products allows for the use of more rapid LC separation techniques [223]. Improvements in sample preparation have come from Immunoaffinity Extraction IAE allowing analyte enrichment and removal of isobaric interferences and matrix effects present in patient serum [220,221,224].

The evolution of the chromatographic methods, mainly based on the type of the coupled detector, for determination of vitamin D and its metabolites can be seen through the contributions of the research team in which the PhD student is integrated. As commented before, the first methods were based on LC–conventional molecular absorption detector with no innovation in the separation–detection step, but in sample preparation [82–86]. Keeping the same chromatographic separation, improvement by post-column derivatization and use of a conventional fluorimetric detector [87] or a laser-induced fluorescence detector [88] was achieved. The coupling of a DAD to the LC [90–93] avoided the derivatization step and provided similar sensitivity with drastic reduction of sample volume and automation of sample

preparation [93], as a prelude of the optimization and application of the LC–MS/MS platform [94–98].

All the previous contributions of the group established the basis for the study in this PhD-Book on the use of SIL-ISs to support quantitative analysis and DEQAS samples for external validation of the method (Chapter 1), and incorporation of two-dimensional LC to MS/MS for inclusion of the C3-epimer-25(OH)D in the analysis of vitamin D metabolites (Chapter 2); sample collection and preparation (Chapter 3), on stability of the target compounds (Chapter 4). Thus, the way for massive application of the final method to the determination of the target compounds in serum or plasma samples from different cross-sectional and longitudinal studies was paved (Chapters 5 and 6).

The use of SIL-IS and validation in LC–MS methods to improve the determination of vitamin D metabolites

SIL-IS are essential for compensating for ionization suppression or enhancement effects and correction of extraction losses during sample preparation.

Isotope dilution-electrospray LC–MS/MS methods performed on ''bench top'' analyzers became popular in the mid-2000s with protein precipitation of the sample, liquid–liquid extraction, short run times, and computer processing of chromatograms contributing to higher throughput and ease of use in the determination of vitamin D and its metabolites [225,226]. Deuterated 25(OHD_2 and D_3 internal standard material improves accuracy and verifies recovery, thereby reducing the problem of ion suppression [227].

Isotope dilution LC–MS/MS is currently considered the gold standard method for 25(OH)D measurement, being able to simultaneously quantificate 25(OH)D_2 and 25(OH)D_3, with summation of the two values resulting in total 25(OH)D.

In the contributions from the PhD student SIL-ISs of both vitamin D_2 and vitamin D_3

and their metabolites were used in all instances (see Chapters 1-to-5).

A review of the International Vitamin D External Quality Assurance Scheme (DEQAS) results for the LC–MS/MS group highlights the spread of results generated by these methods. While the majority of the methods (70–75 %) are positively biased against the all-laboratory trimmed mean (ALTM), some are close to the mean (15–20%) or negatively biased depending on the 25(OH)D concentration measured (5–10%). There has also been concern raised regarding the presence of the 3-epi-25OHD epimer of 25(OH)D, which because of the achiral nature of LC–MS/MS cannot be separated from 25(OH)D by the majority of current methods. The presence of an epimer may increase the total 25(OH)D concentration measured by LC–MS/MS methods compared to immunoassays.

In 2009, the National Institute of Technology (NIST) released SRM 2972, ethanolic 25(OH)D_3 and 25(OH)D_2 calibration solutions to improve standardization of vitamin D assays. SRM 2972 has now been replaced by SRM 2972a 25(OH)D calibration solutions, which contain two ethanolic solutions of 25(OH)D3 as well as single solutions of 25(OH)D_2 and 3-epi-25(OH)D_3 [228].

Validation of the target method has been assessed by the DEQAS program for 5 years, and also the NIST SRM 2972a was used in Chapter 2 for validation of the method for the 25(OH)D_3 epimers.

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HERRAMIENTAS Y EQUIPOS ANALÍTICOS

TOOLS AND ANALYTICAL EQUIPMENT

En este apartado de la Memoria se comentan las características más destacables de las muestras, los aparatos e instrumentos utilizados en el desarrollo experimental de la Tesis, que se describen más detalladamente en los capítulos posteriores.

1. Características de las muestras

Para el desarrollo de la parte experimental que se recoge en el Capítulo 3 de esta Memoria se usaron dos tipos de muestras resultantes obtenidas a partir de sangre: suero y plasma. En las dos matrices se estudió el efecto del pretratamiento de la muestra: la precipitación de proteínas y la extracción en fase sólida, esta última realizada en línea con la separación cromatográfica y la detección subsiguientes. Para la investigación que se recoge en el resto de los Capítulos se utilizó suero como muestra, en la mejora del método —Capítulos 1 y 2—, el estudio de estabilidad de los analitos —Capítulo 4— y de los niveles de los principales metabolitos de la vitamina D_3 del Capítulo 5 y de todos los análisis masivos que aparecen en el Capítulo 6.

2. Sistemas automáticos para la preparación de muestra

En esta Memoria se ha demostrado de forma indiscutible la excelente aplicabilidad de los sistemas automáticos conectados en línea con el conjunto cromatógrafo–espectrómetro de masas para el tratamiento de matrices biológicas en la preparación de la muestra para la determinación de vitamina D y de sus metabolitos. Estos sistemas automáticos llevan a cabo de manera rápida, reproducible y con un consumo mínimo de muestra y de reactivos esta etapa crítica del proceso analítico, disminuyendo al máximo la manipulación por parte del analista, que es una de las principales fuentes de error de los métodos de análisis cuantitativo, especialmente cuando se realizan en gran número. Se utilizó un equipo comercial para llevar a cabo la SPE de forma automatizada: el Symbiosis, que permite trabajar en modo dinámico, llevando a cabo la elución con la fase

móvil cromatográfica. Este dispositivo trabaja a alta presión, posibilitando su conexión en línea con el cromatográfo de líquidos, consiguiéndose así la automatización completa del método analítico. El sistema SPE Symbiosis cuenta con 4 módulos: un automuestreador refrigerado, de enorme interés para las muestras biológicas utilizadas en esta Memoria y especialmente para analitos termoinestables, como los metabolitos de la vitamina D; una unidad de extracción en fase sólida y dos bombas de alta presión dispensadoras de disolventes, de 2 mL de capacidad.

3. Sistemas no automáticos de preparación de muestra

Los sistemas discontinuos se han empleado en el Capítulo 2 de esta Memoria. Dada la naturaleza tanto de la matriz de la muestra como de los analitos de interés, se desarrolló un método con la menor preparación de muestra posible. Esta etapa consistió en la precipitación de proteínas, recogida del sobrenadante, evaporación a sequedad, y reconstitución en el mínimo volumen para alcanzar los bajos niveles de concentración a los que se encuentra los metabolitos en el organismo.

4. Separación y análisis de los metabolitos

Los métodos desarrollados en la parte experimental de esta Tesis Doctoral se han basado en una separación cromatográfica mediante LC y la posterior detección basada en espectrometría de masas. El análisis dirigido, que es el que se utiliza en esta Memoria, se llevó tras la etapa LC, con detección por MS/MS. Se hizo uso de un cromatógrafo Agilent 1200 Series LC equipado con un desgasificador, una bomba binaria y un compartimento de columna termostatizados, y un espectrómetro de masas Agilent 6410 de triple cuadrupolo con una fuente de ionización por electrospray (ESI). En todos los estudios implicando LC, la separación de los analitos se llevó a cabo en una columna cromatográfica de fase reversa Poroshell 120 EC-C18 (50 mm de longitud, 4.6 mm de d.i. y 2.7 μm de tamaño de partícula) de Agilent, acoplada a una precolumna (5 mm de longitud, 4.6 mm de d.i. y 2.1 μm

de tamaño de partícula); excepto en el estudio que recoge el Capítulo 3, para el que se utilizó una columna Pursuit PFP (100 mm de longitud, 4.6 mm de d.i. y 3 μm de tamaño de partícula). Se usaron como fases móviles agua y metanol, a las que se añadió formiato amónico como agente ionizante. En todos los casos se usó el software MassHunter Workstation para la adquisición de espectros y el análisis cualitativo–cuantitativo de los datos.

5. Caracterización del método

En esta Memoria la precisión de los métodos propuestos para el análisis cuantitativo (targeted analysis) se estudió como reproducibilidad dentro del laboratorio y repetibilidad mediante series de experimentos por triplicado usando análisis de varianza (ANOVA) a diferentes niveles de concentración de los analitos. En los Capítulos 4 y 5 el análisis estadístico se realizó mediante el paquete estadístico "stats" del lenguaje de programación R —que permite desarrollar los análisis estadísticos tanto paramétricos como no paramétricos, así como la representación de los datos— y mediante Statgraphics —software que permite realizar distintos análisis estadísticos univariantes y multivariantes.

El primer estudio (Capítulo 1) extendió el número de analitos determinados a la vitamina D (en las dos formas, D_2 y D_3) y sus principales metabolitos: monohidroxilados [$25(OH)D_2$ y $25(OH)D_3$], y dihidroxilados [$1,25(OH)_2D_2$, $1,25(OH)_2D_3$, y $24,25(OH)_2D_3$]. De esta forma quedó drásticamente superado el número de compuestos previamente identificados/cuantificados por el grupo del doctorando y en los estudios existentes en la bibliografía. La cuantificación y validación del método se basó en el uso de patrones marcados isotópicamente (SIL-ISs) y en muestras para la validación externa procedentes de DEQAS, respectivamente.

El segundo estudio de la Sección I se dedicó a la discriminación de los epímeros del $25(OH)D_3$, manteniendo los compuestos incluidos en el Capítulo 1, y a la validación

tanto mediante DEQAS como con un material de referencia certificado del Instituto Nacional de Estándares y Tecnología (NIST-972a). La separación de los epímeros requirió un desarrollo cromatográfico bidimensional previo a la detección mediante MS/MS y tras la separación SPE en línea.

Mejora de las plataformas analíticas para la determinación de vitamina D y sus metabolitos

Improvement of analytical platforms for determination of vitamin D and its metabolites

SECTION I

La Sección I abarca la primera etapa de la investigación realizada, que se dedicó al desarrollo de un método con las características requeridas, para lo que abarcó dos aspectos: uno relacionado con la automatización, la mejora de propiedades analíticas y la validación, y otro dedicado a incrementar el número de metabolitos individualmente identificados/cuantificados. Ambos se desarrollaron con preparación de la muestra mediante SPE, conectada en línea con la separación basada en LC y detección por MS/MS.

El primer estudio en esta sección (Capítulo 1) extendió el número de analitos determinados a la vitamina D (en las dos formas, D_2 y D_3) y sus principales metabolitos: monohidroxilados [$25(OH)D_2$ y $25(OH)D_3$], y dihidroxilados [$1,25(OH)_2D_2$, $1,25(OH)_2D_3$, y $24,25(OH)_2D_3$]. De esta forma quedó drásticamente superado el número de compuestos previamente identificados/cuantificados por el grupo del doctorando y en los estudios existentes en la bibliografía. La cuantificación y validación del método se basó en el uso de patrones marcados isotópicamente (SIL-ISs) y en muestras para la validación externa procedentes de DEQAS, respectivamente.

El segundo estudio de esta Sección I (Capítulo 2) se dedicó a la discriminación de los epímeros del $25(OH)D_3$, manteniendo los compuestos incluidos en el Capítulo 1, y a la validación tanto mediante DEQAS como con un material de referencia certificado del Instituto Nacional de Estándares y Tecnología (NIST-972a). La separación de los epímeros requirió un desarrollo cromatográfico bidimensional previo a la detección mediante MS/MS y tras la etapa de SPE en línea.

Section I contains the first step of the target research, devoted to the development of a method with the required characteristics, for which it encompasses two aspects: one related to automation, sensitivity, selectivity and validation, and the other to increase the number of metabolites to be individually identified/quantified. Both were developed by SPE sample preparation on-line connected with LC separation and MS/MS detection.

The first of the research in this section (Chapter 1) expanded the number of determined analytes to vitamin D (both D_2 and D_3) and its main metabolites: monohydroxylated [25(OH)D_2 and 25(OH)D_3], and dihydroxylated metabolites [1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$]. In this way, the number of compounds previously identified/quantified by the group of the PhD-student and those of the methods existing in the literature were drastically surpassed. Quantitation and validation of the method was supported on the use of stable isotopic labeled standards (SIL-ISs), and samples from the vitamin D External Quality Assurance Scheme (DEQAS), respectively.

The second of the research included in Section I (Chapter 2) dealt with discrimination between 25(OH)D_3 epimers (keeping the compounds in Chapter 1) and validation by both DEQAS and a standard reference material certified by the National Institute of Standards and Technology (NIST-972a). Epimers separation required two-dimensional LC subsequent to on-line SPE separation, and prior to MS/MS detection.

Capítulo 1

Quantitative analytical method to evaluate the metabolism of vitamin D

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Quantitative analytical method to evaluate the metabolism of vitamin D

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Abstract

A method for quantitative analysis of vitamin D (both D_2 and D_3) and its main metabolites —monohydroxylated vitamin D (25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3), and dihydroxylated metabolites (1,25- dihydroxyvitamin D_2, 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3)— in human serum is here reported. The method is based on direct analysis of serum by an automated platform involving on-line coupling of a solid-phase extraction workstation to a liquid chromatograph–tandem mass spectrometer. Detection of the seven analytes was carried out by the selected reaction monitoring (SRM) mode, and quantitative analysis was supported on the use of stable isotopic labeled internal standards

(SIL-ISs). The detection limits were between 0.3–75 pg/mL for the target compounds, while precision (expressed as relative standard deviation) was below 13.0% for between-day variability. The method was externally validated according to the vitamin D External Quality Assurance Scheme (DEQAS) through the analysis of ten serum samples provided by this organism. The analytical features of the method support its applicability in nutritional and clinical studies targeted at elucidating the role of vitamin D metabolism.

Keywords: Vitamin D, Metabolites, LC–MS/MS, Automation, Serum, DEQAS

1. Introduction

The vitamin D endocrine system (VDES) plays an essential role in human health beyond the regulation of calcium homeostasis and bone mineralization [1]. Vitamin D is obtained as vitamin D_3 (cholecalciferol) mainly derived from photosynthesis in the skin, after conversion of 7- dehydrocholesterol by ultraviolet irradiation. Small amounts of vitamin D can also be obtained by nutritional intake of either vitamin D_3 or by foods fortified with vitamin D_2 (ergocalciferol). Both forms undergo a substrate-dependent liver hydroxylation to 25-hydroxyvitamin D [25(OH)D_2 and 25(OH)D_3], the major circulating form of vitamin D considered a reliable indicator of its nutritional status [2], despite the recognized lack of assay standardization for its determination [3]. To be fully active, 25(OH)D must further be converted to 1,25- dihydroxyvitamin D [1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$], the hormonally active form of VDES, and under whose control is considered to be around 3% of the human genome [4]. The recent growing interest in VDES is mainly due to its multiple roles in global health maintenance. Apart from classical diseases such as rickets, osteomalacia and osteoporosis, vitamin D insufficiency has been associated to several chronic diseases (*e.g.*, arthritis, cardiovascular, cancer, diabetes, multiple sclerosis, psychiatric illnesses) [5], thus increasing nearly exponentially over the last decade the demand from basic and clinical scientists for tests intended to quantify vitamin D and metabolites [5]. The essential question of how much vitamin D is needed for optimal bone and global health remains unsolved [6,7]. Despite vitamins D_2 and D_3 are generally considered to be equivalent in humans, there are evidences that vitamin D_3 is substantially more efficient than vitamin D_2 to rise serum 25(OH)D concentrations [8,9]. The analysis of vitamin D is complicated by the structural similarity of metabolites, hydrophobic nature and thermal and UV-light instability [10]. In fact, there are over 40 identified vitamin D (D_2/D_3) metabolites [11], among which, the targets of clinical analyses are 1,25$(OH)_2D_3$ and 25(OH)D_3 as indicators of vitamin D nutritional status [2,6–9,12]. Measurement of 1,25$(OH)_2D_3$ is more challenging

than that of 25(OH)D_3 as the former is more unstable and it is present in serum/plasma at the pg/mL level versus ng/mL level for 25(OH)D_3. Current methods for determination of 25(OH)D and 1,25(OH)2D include competitive protein binding assay (CPB) [13], Radioimmunoassay (RIA) [14,15], Enzyme-Linked Immuno-Sorbent Assay (ELISA) [16], and Chemiluminescence Immunoassays (CLIA) [13], which have demonstrated cross-reactivity for 25(OH)D_3/25(OH)D_2 and 1,25(OH)$_2D_3$/24,25(OH)$_2D_3$ pairs [5,17]. Despite liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods are at present considered the "gold standard" [3,18–20], they have already aroused controversy [21,22], which requires in depth research to achieve more consistent results [3]. A characteristic of the present LC–MS/MS methods for vitamin D analysis including 1,25(OH)2D as target analyte when applied to serum or plasma is the time-consuming and tedious sample preparation step. Preparation of serum/plasma samples for determination of the dihydroxymetabolite makes mandatory its preconcentration and removal of interferents. Automated solid phase extraction (SPE) constitutes an excellent tool to facilitate and speed up the steps involved in sample preparation. In addition, the system can be on-line coupled to LC equipment thus allowing monitoring of the entire SPE process with recovery estimation [20]. Apart from automation, minimization of matrix effects and other variability sources is crucial to improve the overall method. The use of stable isotopic labeled internal standards (SIL-ISs) [23–25] with physico-chemical properties identical or similar to the target analytes is critical to overcome variability sources [26]. Proficiency testing programs have been created to assess comparative performances and improve the accuracy of total 25(OH)D and 1,25(OH)2D methods [3,27]. Among them, the Vitamin D External Quality Assessment Scheme (DEQAS) is presently considered an accuracy-based program for selected vitamin D metabolites [28].

Despite present methods based on LC–MS/MS are most times endowed with excellent sensitivity and selectivity, there is a demand for more robust, fully

automated platforms that can meet the need for throughput, precision and accurate testing of vitamin D and metabolites with special emphasis on the dihydroxymetabolites due to their low physiological concentrations. The present research was aimed at validating an automated SPE–LC–MS/MS platform with optimum analytical features for determination of vitamin D and the most important metabolites in the clinical and nutritional fields. Apart from vitamins D_2 and D_3, the method was targeted at the monohydroxymetabolites of the two forms of vitamin D as well as the three dihydroxymetabolites: $1,25(OH)_2D_2$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The inclusion of vitamin D_2 and metabolites in analytical methods is not usual as this form is practically not detected in humans unless it is administered as supplement, which is a common practice in USA. It is worth emphasizing that the development of the method was supported on the use of SIL-IS for the different analytes and the validation was carried out with an external quality assessment (DEQAS).

2. Experimental

2.1. Chemicals and reagents

LC–MS grade reagents and solvents were used in this research. Ammonium formate from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA) and acetonitrile, formic acid and methanol from Scharlab (Barcelona, Spain) were used for preparation of chromatographic mobile phases and SPE solutions.

Metabolites $1,25(OH)_2D_2$, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $25(OH)D_2$, $25(OH)D_3$, vitamin D_2 and vitamin D_3 were from Sigma. Stable isotopic standards $1,25(OH)_2D_3$-d_6, $24,25(OH)_2D_3$-d_6, $25(OH)D_3$-d_6, and vitamins D_3-d_6 were synthesized by A. Mouriño and M. A. Maestro (vitamin D research group). The $25(OH)D_2$-d_3 and vitamins D_2-d_3 isotopic standards were from Sigma. Individual standard solutions were prepared by dissolving 1 mg of each analyte or isotopic standard in 10 mL of methanol, from which two solutions used for spiking the

biological samples (either serum or plasma) were prepared by dilution of the appropriate volume in methanol. One multistandard working solution was prepared with the target analytes at different concentrations: 100 ng/mL for dihydroxymetabolites 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$; 1 µg/mL for 24,25$(OH)_2D_3$; 5 µg/mL for 25$(OH)D_3$ and 25$(OH)D_2$; and 10 µg/mL for vitamins D_2 and D_3. Other solution was prepared with each isotopic standard —7.5 ng/mL for isotopic standard 1,25$(OH)_2D_3$-d_6 and 125 ng/mL 24,25$(OH)_2D_3$-d_6; 625 ng/mL for 25$(OH)D_3$-d_6 and 25$(OH)D_2$-d_3; and 625 ng/mL for vitamin D_2-d_3 and vitamin D_3-d_6.

2.2. Instruments and apparatus

The analyses were performed by reversed-phase LC (RP-LC) separation followed by electrospray ionization in positive mode (ESI+) and MS/MS detection. Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer. The data were processed using MassHunter Workstation Software (V-B.05) for qualitative and quantitative analyses. Hyphenated SPE was performed by a Symbiosis system (Spark Holland, Emmen, The Netherlands). This commercial equipment comprises a unit for automatic cartridge exchange (ACE), an autosampler (Reliance) furnished with a 0.2 mL sample loop and two high-pressure syringe dispensers (HPDs) for SPE solvent delivery. Peek tubing of 0.25 mm i.d. (VICI, Houston, Texas, USA) was used to connect all valves of the Symbiosis unit and LC–MS/MS modules. A stainless tube of 1.0 mm i.d. and 130 cm length, about 1 mL volume, was used to connect the Reliance to the ACE unit for mixing the serum sample and loading solution. A 10×2 mm cartridge packed with Hysphere C8 (Spark Holland) as sorbent material was used for SPE. The analytical column was a Poroshell 120 EC-C18 (2.7 µm particle size, 50×4.6 mm i.d.) from Agilent, while a guard column (2.7 µm particle size, 5.0×2.1 mm i.d.), also from Agilent, was used to preserve the integrity of the analytical column.

2.3. Sampling, sample collection and storage

Ten serum samples were provided by DEQAS for analysis of 25(OH)D and 1,25(OH)2D using the proposed method. The samples were prepared from individual blood donations and were sterilized by filtration through 0.2 μm microbiological grade filters by the Oncology/Endocrinology Laboratory of Charing Cross Hospital (Fulham Palace Rd, London W6 8RF, UK) [28].

Three serum pools were prepared by spiking the target analytes at low, intermediate and high concentrations according to the physiological levels described in the literature [29]. The concentrations used for preparation of each pool are listed in Supplementary Table 1. The pools were used for evaluation of analytical features such as precision, recovery factor for the SPE step and accuracy of the method.

2.4. Proposed procedure

A volume of 240 μL of serum in an amber glass vial was spiked with 10 μL of the deuterated working solution —final concentration in serum: 25 ng/mL of vitamin D_3-d_6 and vitamin D_2-d_6, 25 ng/mL of 25(OH)D_3-d_6 and 25(OH)D_2-d_6, and 5 ng/mL of 24,25$(OH)_2D_3$-d_6 and 0.3 ng/mL of 1,25$(OH)_2D_3$-d_6— shaken and introduced into the autosampler. Supplementary Fig. 1.A shows the analytical platform used for determination of vitamin D and its metabolites. The sample loop was filled with 0.2 mL of filtered serum from the sample vial, which was refrigerated in the Reliance unit at 6 °C. The serum was subjected to the sequence of automatic operations described in Supplementary Table 2.

The initial chromatographic mobile phase was 5 mM ammonium formate in 85:15 (v/v) methanol–water at a flow rate of 0.3 mL/min. The temperature in the column compartment was 15 °C. At 2 min, a linear gradient was programmed to obtain 5 mM ammonium formate in methanol for 5 min. The final gradient

conditions were maintained for 10 min until the end of the chromatographic separation step. The total analysis time was 15 min, 10 additional min being required for re-establishing and equilibrating the initial conditions. A last step to purge tubing and cartridge for reuse was developed in the Symbiosis system. The chromatographic detection step of a sample and the SPE step of the next sample overlapped, thus improving the analysis frequency.

The eluate from the chromatographic column was monitored by MS/MS in selected reaction monitoring (SRM). The flow and temperature of the drying gas (N_2) were 9 L/min and 350 °C, respectively. The nebulizer pressure was 50 psi, and the capillary voltage 4750 V in positive mode.

2.5. Data treatment

Quantitation was carried out using the ratio between the peak area of each analyte and that of the corresponding SIL-IS. Quantitative analysis of $1,25(OH)_2D_2$ was carried out by using $1,25(OH)_2D_3$ as IS due to their structural similarity.

A calibration model was developed for each analyte using a serum pool, aliquots of which were spiked with different concentrations of the standard of the target analyte and a constant concentration of the deuterated standard. Also, a blank serum spiked only with deuterated standards was prepared to correct the endogenous concentration of the target analyte in the serum pool.

3. Results and Discussion

3.1. LC–MS/MS detection by SRM

The sequence followed in the optimization of MS/MS detection was as follows: isolation of the precursor ion with the highest efficiency; study of the product ions; and, finally, selection of the most sensitive and selective transition from each precursor ion to the most suitable product ions. Table 1 and Supplementary Table

3 list the information for quantitative analysis of the target analytes and SIL-ISs with precursor and product ions, dwell time used for each SRM transition, collision energy values and the transitions selected for quantitative purposes. As can be seen, precursor ions for each analyte/deuterated pair reported a mass shift of 6 m/z units. MS scans for each analyte/deuterated pair are illustrated in Supplementary Fig. 2.

Table 1. Optimization of the MS/MS SRM method for quantitative and confirmatory analysis of vitamin D and metabolites.

Analyte	Precursor ion (m/z)	Adducts	Product ion (m/z)	Dwell time (ms)	Collision energy (V)	Quantitation transition	Retention time (min)	Internal standard
$24,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	295.1	30	15	399.3→121.1	3.7	$24,25(OH)_2D_3$-d_6
			159.0	30	25			
			121.1	30	20			
$1,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	161.1	30	25	399.3→147.1	4.5	$1,25(OH)_2D_3$-d_6
			147.1	30	30			
			107.0	30	30			
$1,25(OH)_2D_2$	411.3	$[M+H-(H_2O)]^+$	285.1	45	21	411.3→133.1	5.0	$1,25(OH)_2D_3$-d_6
			133.1	45	21			
$25(OH)D_3$	383.3	$[M+H-(H_2O)]^+$	365.3	52	10	383.3→159.1	7.5	$25(OH)D_3$-d_6
			173.1	52	25			
			159.1	52	25			
$25(OH)D_2$	395.3	$[M+H-(H_2O)]^+$	377.0	52	10	395.3→133.1	7.7	$25(OH)D_2$-d_3
			199.0	52	25			
			133.1	52	30			
Vit D_2	397.3	$[M+H]^+$	379.2	52	5	397.3→107.0	11.7	Vit D_2-d_3
			159.1	52	25			
			107.1	52	28			
Vit D_3	385.3	$[M+H]^+$	259.2	52	10	385.3→107.1	12.2	Vit D_3-d_6
			147.1	52	25			
			107.1	52	30			

Additionally, it is worth mentioning that the monitored product ions for each vitamin D_3 metabolite and its corresponding deuterated standard were equal. Therefore, product ions should fit molecular structures without deuterium atoms. This is not a limitation since quantitative transitions were clearly different for analytes and ISs. Figures 1 and 2 show MS/MS spectra obtained for each precursor ion with the most representative product ions monitored in quantitative and qualitative transitions. The spectra were obtained after LC–MS/MS analysis of

multistandard solutions of the target analytes and SIL-ISs. Detection of qualitative transitions was required to confirm identification of all analytes.

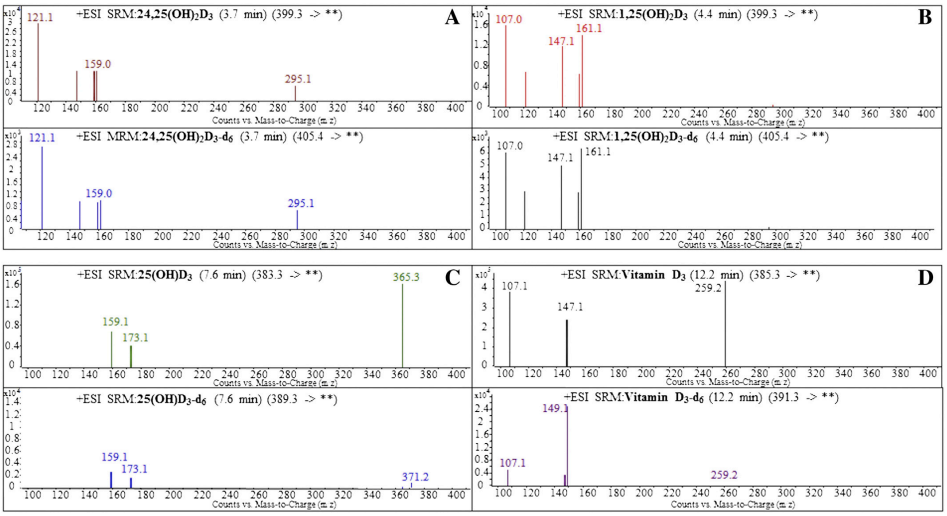

Fig. 1. MS/MS spectra of vitamin D_3, its metabolites and their SIL-ISs: (A) 24,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$-d_6; (B) 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-d_6; (C) 25$(OH)D_3$ and 25$(OH)D_3$-d_6; (D) vitamin D_3 and vitamins D_3-d_6.

3.2. Optimization of the SPE step

Sensitivity in the analysis of vitamin D is a crucial analytical feature taking into account the low levels at which relevant metabolites such as the dihydroxy-metabolites are present in serum. The complexity of a biofluid as serum is a limitation to achieve low detection limits by analysis of fractions obtained by liquid–liquid extraction with non-aqueous solvents. This limitation is ascribed to ionization suppression effects that contribute to decrease the sensitivity of LC–MS/MS methods. In this context, sample preparation based on extraction of the

target analytes and cleanup for interferents removal is the appropriate strategy. For this purpose, SPE seems to be the best approach taking benefits from the selectivity of sorbent–analyte interactions.

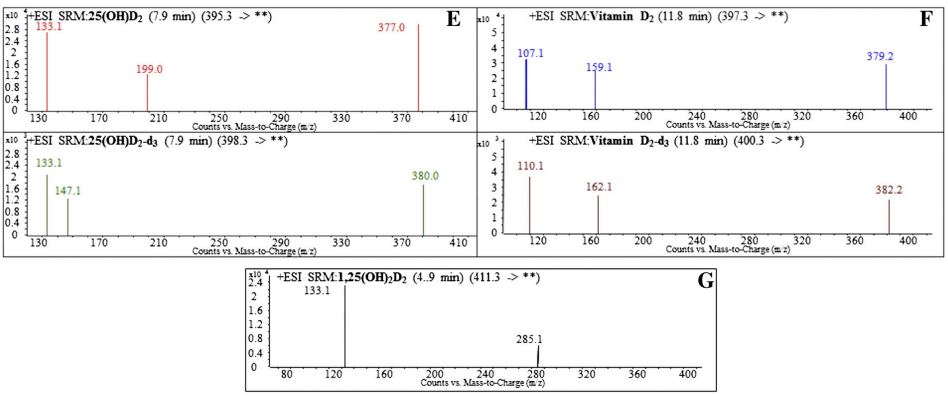

Fig. 2. MS/MS spectra of vitamin D_2, its metabolites and their SIL-ISs: (E) 25(OH)D_2 and 25(OH)D_2-d_3; (F) vitamin D_2 and vitamins D_2-d_3; (G) 1,25(OH)$_2D_2$.

Despite the similarity of the chemical structures of vitamin D and metabolites, critical differences in the interaction with sorbents can be found. Thus, vitamin D, 25(OH)D_3, and 1,25(OH)$_2D_3$ could interact differently with SPE sorbents. A previous study identified C18 and C8 SPE sorbents as the most suited materials for extraction of vitamin D and metabolites as well as for interferents removal [30]. Both sorbents are based on different degrees of lipophilic interactions. In the present study, the two SPE sorbents were evaluated to retain vitamin D and metabolites. The SPE optimization was carried out with aliquots of a serum pool spiked with standard solutions of the target analytes —final concentrations in the pool: 100 ng/mL for vitamins D_2 and D_3, 50 ng/mL for 25(OH)D_3 and 25(OH)D_2, and 10 ng/mL for 1,25(OH)$_2D_2$, 1,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$. The sample

volume was set at 200 µL to decrease detection limits up to values demanded for analysis of clinical samples. Figure 3 shows the extracted ion chromatograms (EICs) obtained with C8 and C18 sorbents for vitamin D_3, 25(OH)D_3 and 1,25$(OH)_2D_3$ as representative metabolites of the three groups of analytes monitored in this research: vitamin D, mono- and dihydroxylated metabolites. As can be seen, C18 showed the best retention/elution behavior for vitamin D_3, justified by the higher lipophilic interactions with the C18 sorbent as compared to C8. On the other hand, C8 was clearly the best sorbent for 25(OH)D_3 and 1,25$(OH)_2D_3$. Taking into account that the analysis of vitamin D is compromised by the sensitivity of hydroxylated metabolites and, particularly, of dihydroxylated metabolites, the C8 sorbent was selected for further studies.

The variables subjects of the optimization study are listed in Supplementary Table 4 with detailed information of the experiments carried out in each step: sample loading, sorbent washing-up and elution. Supplementary Fig. 3 shows the SRM chromatograms obtained by analysis of serum spiked at 100 ng/mL for vitamins D_2 and D_3, 50 ng/mL for monohydroxymetabolites and 10 ng/mL for dihydroxymetabolites.

3.3. Analytical characteristics of the method

3.3.1. Sensitivity and linear calibration range

Calibration plots were run using the peak area ratio between the target analyte and that of the IS as a function of the standard concentration of each compound. Regression coefficients were above 0.99 for all calibration models. The analytical features of the calibration models are shown in Table 2. The limits of detection (LOD) and quantitation (LOQ) for each analyte were calculated as the concentration providing signals three and ten times, respectively, higher than the background noise measured at a time close to each chromatographic signal.

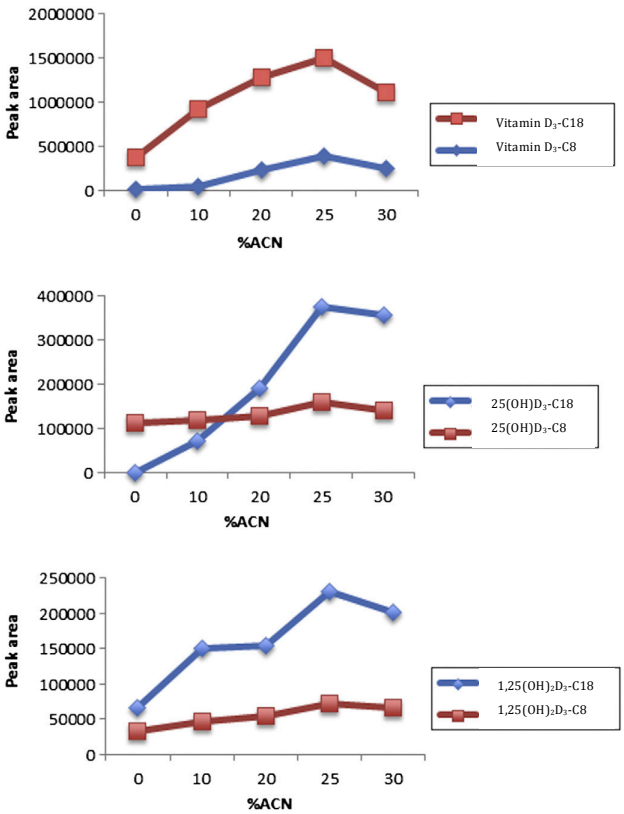

Fig. 3. Influence of the loading solvent composition (acetonitrile (ACN)–water) on the retention efficiency of three representative analytes: vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ using C8 and C18 SPE sorbents.

As Table 2 shows, the sensitivity was better for vitamin D_3 and metabolites as compared to vitamin D_2. In the former case the LOQ values were below the normal concentrations observed in human serum, according to the literature [31,32]. It is worth noting that the two vitamin D_3 metabolites, $25(OH)D_3$ and $1,25(OH)_2D_3$, which are the circulating and active forms of vitamin D_3, respectively, reported low LOQ values (1.2 and 5.0 pg/mL, respectively). Therefore, the status of vitamin D_3

can be well evaluated by the proposed method since normal levels of both metabolites are clearly above the LOQ values [29].

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3.3.2. Evaluation of the precision of the method

The precision of the method was evaluated by calculation of the within-day variability and between-days variability both expressed as RSD. For this purpose, a single experimental set-up with duplicate analysis per day (for 7 days) was carried out with a serum pool spiked with the target analytes at intermediate levels (Supplementary Table 1) according to physiological levels described in the literature [29]. The obtained results, listed in Table 2, show that precision,

expressed as RSD, was below 13%, which proves the efficiency of the SPE step for cleaning and preconcentration of the target analytes. The within day variability was below 4.5% for all analytes, except for $1,25(OH)_2D_2$ and vitamin D_3, which reported 8.1 and 11.5%, respectively.

Table 2. Analytical characteristics of the method.

Analyte	LOD (pg/mL)	LOQ (pg/mL)	Calibration range	Equation	Regression coefficient	Within-day variability (%)	Between-days variability (%)
$24,25(OH)_2D_3$	1.5	5	5 pg/mL –50 ng/mL	y = 1.794x – 0.564	0.992	1.5	2.6
$1,25(OH)_2D_3$	1.5	4	4 pg/mL – 5 ng/mL	y = 0.0013x + 0.144	0.996	2.9	9.2
$1,25(OH)_2D_2$	15.1	50	50 pg/mL– 50 ng/mL	y = 2.065x – 0.425	0.998	8.1	9.0
$25(OH)D_3$	0.3	1.2	1.2 pg/mL –250 ng/mL	y = 0.514x – 0.427	0.997	4.5	8.4
$25(OH)D_2$	75.7	250	0.25 ng/mL –250 ng mL	y = 0.622x – 0.548	0.996	2.0	3.5
Vit D_2	15.1	50	50 pg/mL –500 ng/mL	y = 0.171x – 0.473	0.990	4.0	7.2
Vit D_3	0.3	1	1 pg/mL–500 ng/mL	y = 1.276x –1.5581	0.996	11.5	13.0

3.3.3. Recovery factor

The recovery factor was calculated using the three pools of human serum spiked at low, intermediate and high concentrations of the target analytes according to physiological levels described in the literature (Supplementary Table 1) using the two cartridges configuration of the SPE workstation (illustrated in Supplementary Fig. 1.B) [33–35]. In this configuration, two cartridges with the same sorbent are put in serial; hence the retention capability of the C8 SPE sorbent is evaluated by comparison of the amount of analyte retained in both cartridges. With this configuration, the recovery factor was calculated as amount of analyte retained in cartridge 1/[amount of analyte in cartridge 1+amount of analyte in cartridge 2]. The values obtained for the three spiked levels allowed obtaining a medium recovery factor for each analyte, which is shown in Table 3. The recovery factor was practically 100% for dihydroxymetabolites, which is crucial for their quantitation in serum. On the other hand, the recovery factors for monohydroxymetabolites of D_3 and D_2 were 98.8 and 97.2%, respectively, while the recovery factors decreased up to 59.1 and 60.2% for vitamin D_2 and vitamin D_3,

respectively. The decreased polarity of vitamin D led to a decrease of the SPE efficiency of the C8 sorbent. It is worth emphasizing that lipophilic interactions of the C18 sorbent were more suited for retention/elution of vitamin D_3 and vitamin D_2 than those observed for C8, which justifies the lowest recovery factors for both analytes. The next step was focused on the accuracy study to check if internal standards are able to correct retention efficiency anomalies.

3.3.4. Accuracy of the method

The accuracy of the method and potential matrix effects were studied for each analyte by analysis of the three serum pools spiked at three different concentrations depending on the analyte (Supplementary Table 1).

Table 3. Estimation of recovery for the SPE step and accuracy of the method for quantitative analysis of each analyte.

Analyte	Recovery factor[1] (%)	Accuracy[2] (%)
$24,25(OH)_2D_3$	100	89.3
$1,25(OH)_2D_3$	100	95.9
$1,25(OH)_2D_2$	99.8	90.9
$25(OH)D_3$	98.8	96.3
$25(OH)D_2$	97.2	89.4
Vit D_2	59.1	83.3
Vit D_3	60.2	93.6

Non-spiked serum aliquots were also analyzed (three replicates/aliquot) to compare the concentrations spiked with those calculated by the calibration models. For this purpose, the one-cartridge configuration was used (Supplementary Fig. 1.A). The accuracy was calculated by the ratio (measured concentration–endogenous concentration)/spiked concentration. According to this equation, vitamins D_2 and D_3 reported average accuracy values (by considering the

values obtained by analysis of the three serum pools) of 83.3 and 93.6%, respectively. Therefore, the internal standard used for their quantitative analysis allowed compensating errors in the recovery factors. The sensitivity of the method for these analytes is enough for their quantitation in human serum. The average accuracy for dihydroxylated metabolites ranged from 89.3 to 95.9%, which are within the precision range previously estimated. Finally, the monohydroxylated metabolites reported values of 96.3 and 89.4% for 25-hydroxyvitamins D_3 and D_2, respectively. The accuracy of the method guarantees quantitation of vitamin D and its main five metabolites with capability to evaluate the metabolic status of this liposoluble vitamin.

3.4. External validation of the method according to DEQAS

The method was externally validated according to the DEQAS program. For this purpose, ten samples (code number 451–460) provided by DEQAS were analyzed in triplicate to determine the concentration of total 25(OH)D, as the sum of concentrations of 25(OH)D_3 and 25(OH)D_2, which was compared for each sample with the corresponding target value provided by DEQAS. The reference values used for comparison correspond to the average concentrations obtained by considering only those laboratories using LC–MS/MS methods, but also all the different laboratories ascribed to the DEQAS program, which involved the application of five different analytical techniques. Supplementary Table 5 lists the average concentrations provided by (i) the proposed method, (ii) all LC–MS/MS methods, and (iii) All-Laboratory Trimmed Mean (ALTM). Also the bias of the proposed method related to the LC–MS/MS methods and ALTM methods, expressed as percentage, are in the table. The bias percentage by taking the values provided by all laboratories using LC–MS/MS as reference concentrations ranged from 2.2 to 9.5%, which involved an accuracy always above 90% for this metabolite.

The bias percentage by using the average concentrations of all laboratories ranged from1.8 to 10.5% for all samples, except for sample 453 that gave 18%. According to DEQAS specifications, external validation is passed if this percentage of bias is below25%, which was fulfilled by this set of samples. A correlation analysis between concentrations provided by the proposed SPE–LC–MS/MS and the DEQAS target values provided a coefficient of correlation (R^2) of 0.9465, which supports the external validation for this parameter (Supplementary Fig. 4).

Other ten additional samples (code number 341–350) were analyzed in triplicate to determine the total concentration of 1,25(OH)2D [$1,25(OH)_2D_3$ + $1,25(OH)_2D_2$]. The obtained results were compared with the target values provided by those laboratories using LC–MS/MS methods and by all laboratories ascribed to DEQAS, as Supplementary Table 5 shows. As can be seen, the percentage of bias by comparison with LC–MS/MS laboratories ranged from 0.4 to 14.8%, except for two samples (346 and 347) that the method here reported overestimated values around 20% (25.8 and 19.4, respectively). Nevertheless, the comparison with the average values corresponding to all laboratories ascribed to DEQAS led to better accuracy results since the bias percentage was always below 11%, except for sample 349 that provided 15.3%. These values are within the limits established by DEQAS, set at ±30% in this case, taking into account that this metabolite is generally detected at pg/mL in serum. By analogy, the correlation analysis provided an R^2 value of 0.8853, which also proves a good performance of the proposed method (Supplementary Fig. 4). In general terms, the SPE–LC–MS/MS method provided an overestimation of the levels of 25(OH)D and 1,25(OH)2D as compared to the values provided by all laboratories but, in any case, the variability estimated was within the limits imposed by DEQAS. Additionally, it is worth mentioning that the variability estimated for each sample was within the precision values listed in Table 2.

4. Conclusions

An automated method for quantitative analysis of vitamin D and its main metabolites has been here validated. The method was designed to provide a fast chromatographic separation of vitamins D_3 and D_2 and their main metabolites [$1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$, $25(OH)D_3$, $25(OH)D_2$] in serum samples. The use of SIL-ISs endows the method with a suitable tool for avoiding or minimizing ionization suppression effects, and compensates for other variability sources induced by sample preparation steps (*e.g.*, dilution, concentration by solvent evaporation and/or analytes adsorption, instrumental variations including injection volume and ionization effects, as well as variations in response over time).

The method is very appropriate to determine the 25(OH)D metabolite, which is that usually evaluated in most clinical analysis as it is the indicator of the nutritional state of vitamin D. In the countries where supplementation (either nutritional or pharmacological) is provided by vitamin D_2, both vitamins D_2 and D_3 should be analyzed as a contribution of the latter as cutaneous synthesis always exists.

The method is also very appropriate to determine the $1,25(OH)_2D$ metabolite, the analysis of which is presently very demanded in patients with renal insufficiency, hypoparathyroidism and hyperparathyroidism, and also for screening of hyper-calcemia, increase of extra renal synthesis, etc. Despite quantitation of the $24,25(OH)_2D$ is not at present relevant in routine clinical analysis, it could be very useful for clinical research to define the cut-off of vitamin D status, of great present controversy. The sensitivity of the proposed method could help in basic research on in vivo and in vitro models.

Despite quantitation of vitamins D_2 and D_3 is not necessary from the clinical point of view owing to fast conversion into the corresponding 25(OH)D metabolite,

methods to differentiate both forms are of interest in pharmacokinetics studies, and to know the absorption capacity of metabolites orally administrated. Also, the determination of both vitamin D_2 and its 25(OH)D_2 metabolite could help in the study of the capability of vitamin D absorption in malabsorption processes as celiac disease, inflammatory bowel disease or bariatric surgery. On the other hand, determination of vitamin D_3 is useful to evaluate the ability of synthesis of this vitamin by ultraviolet irradiation.

With these premises, the next step for assessment is the application of the method to an extended cohort recruited in clinical centers.

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Supplementary Table 1. Concentrations of the target analytes spiked in human serum for estimation of the recovery factor and for the accuracy study.

Analyte	Low level (ng/mL)	Intermediate level (ng/mL)	High level (ng/mL)
$1,25(OH)_2D_3$	0.05	0.1	0.2
$1,25(OH)_2D_2$	3	10	20
$24,25(OH)_2D_3$			
$25(OH)D_3$	15	50	100
$25(OH)D_2$			
Vit D_2	15	50	100
Vit D_3			

Supplementary Table 2. Concentrations of the target analytes spiked in human serum for estimation of the recovery factor and for the accuracy study.

Step	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				
Start autosampler				Load sample
Solvation 1	10	2	Methanol	
Solvation 2	5	4	Methanol	
Equilibration	3	4	25% ACN–0.7%F	
Equilibration	3	4	25% ACN–0.7%F	
Sample application	3	4	25% ACN–0.7%F	
Wash cartridge	3	0.5	30% ACN	
Elution			Mobile phase	5 min
Purge 1	5	5	Methanol	
Purge 2	5	5	30% ACN	
Purge 3	5	5	Water	
Purge 4	5	5	30% ACN	
Purge 5	5	5	Water	

Supplementary Table 3. Optimization of the MS/MS SRM method for quantitative and confirmatory analysis of SIL-IS.

Analyte	Precursor ion (*m/z*)	Adducts	Product ion (*m/z*)	Dwell time (ms)	Collision energy (V)	Quantitation transition	Retention time (min)
24,25(OH)$_2$D$_3$-d$_6$	405.4	[M+H-(H$_2$O)]$^+$	295.1	30	15	405.4→121.1	3.7
			159.0	30	25		
			121.1	30	20		
1,25(OH)$_2$D$_3$-d$_6$	405.4	[M+H-(H$_2$O)]$^+$	161.1	30	25	405.4→147.1	4.5
			147.1	30	20		
			107.0	30	35		
25(OH)D$_3$-d$_6$	389.3	[M+H-(H$_2$O)]$^+$	365.3	39	10	389.3→159.1	7.5
			173.1	39	30		
			159.1	39	25		
25(OH)D$_2$-d$_3$	398.3	[M+H-(H$_2$O)]$^+$	380.0	25	20	389.3→147.1	7.7
			147.1	45	30		
			133.1	25	25		
Vit D$_2$-d$_3$	400.3	[M+H]$^+$	382.2	25	10	400.3→110.0	11.7
			162.1	25	20		
			110.0	40	20		
Vit D$_3$-d$_6$	391.3	[M+H]$^+$	259.2	50	10	391.3→107.1	12.2
			147.1	50	25		
			107.1	50	20		

Supplementary Table 4. Optimization of the automated SPE step for analysis of vitamin D and metabolites.

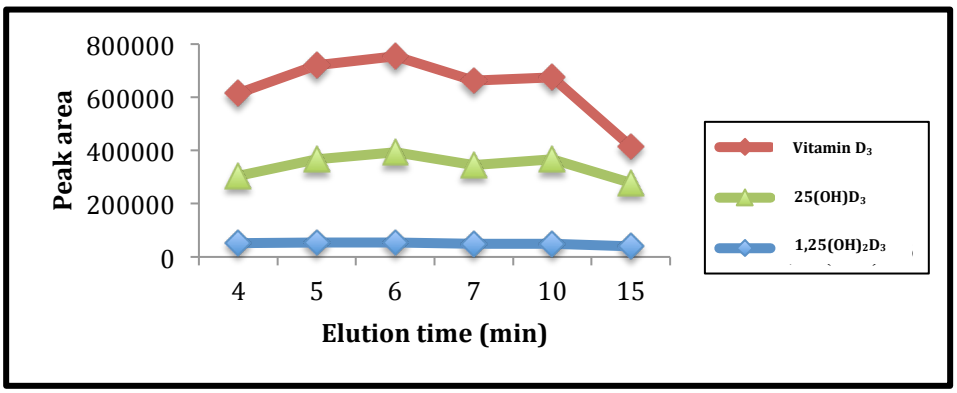
Variable	Tested variable	Optimum
SPE sorbent	C8, C18	C8
Sample volume (µL)	50–200	200
Loading solvent	0:100–40:60 methanol–water (0–1% FA) 0:100–40:60 acetonitrile–water (0–1% FA) 0.5–6 mL 0.5–3 mL/min	25:75 acetonitrile–water 0.7% FA 4 mL 3 mL/min
Washing solvent	0:100–40:60 methanol–water 0:100–40:60 acetonitrile–water 0.5–6 mL 0.5–3 mL/min	30:70 acetonitrile–water 0.5 mL 3 mL/min
Elution time (min)	4–15	6

Sample loading was optimized by testing different solvent compositions using methanol–water and acetonitrile–water mixtures. The concentration of organic solvent was tested from 0 to 30% (v/v) because higher concentrations led to precipitation of proteins in the

tubes. Acetonitrile was selected as organic solvent because retention of the target analytes was clearly enhanced as compared to the use of methanol. Concerning the loading solvent, percents of acetonitrile in water between 0 and 30% were checked. Technical limitations impeded to test concentrations higher than 30% (v/v) of acetonitrile because of protein precipitation in the tubing system. A 25% (v/v) acetonitrile in water was the optimum composition to maximize the retention capability of the C8 sorbent. The pH of this solution was studied by using concentrations of formic acid within the range 0.1–0.9% (v/v) with the purpose of releasing vitamin D and metabolites bound to transporting proteins. Formic acid concentrations 0.7 or 0.9% (v/v) enhanced the analytical responses of 25(OH)D_3 and 1,25$(OH)_2D_3$ since moderate acid pH values favor the release of vitamin D metabolites from transport proteins and, additionally, hydrophobic interactions are enhanced under these conditions. Therefore, 0.7% (v/v) formic acid was used in the loading step.

Concerning the washing step, different methanol–water and acetonitrile–water mixtures from 0:100 to 30:70 (v/v) were evaluated to minimize ionization suppression effects. Acetonitrile solutions proved more efficient than methanol mixtures, and 30:70 (v/v) acetonitrile–water was the optimum washing solution since higher concentration of organic solvent led to partial elution of the target analytes. The optimum volume for washing the cartridge was 0.5 mL at 3 mL/min.

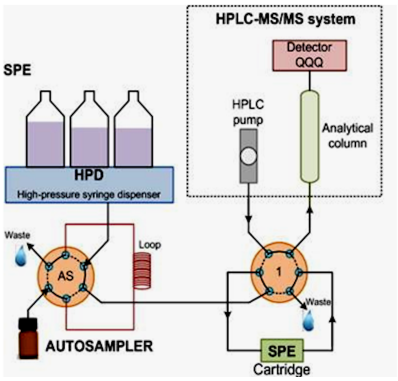
Finally, the elution step was studied by testing different elution times from 4 to 15 min during which the chromatographic mobile phase was pumped through the C8 sorbent. The elution time was studied to avoid elution of matrix interferents from the cartridge that could produce ion suppression in the QqQ MS/MS detector. As the figure below shows, the area of the peaks increased up to an elution time of 6 min, while longer values lead to peak broadening effects by dispersion.



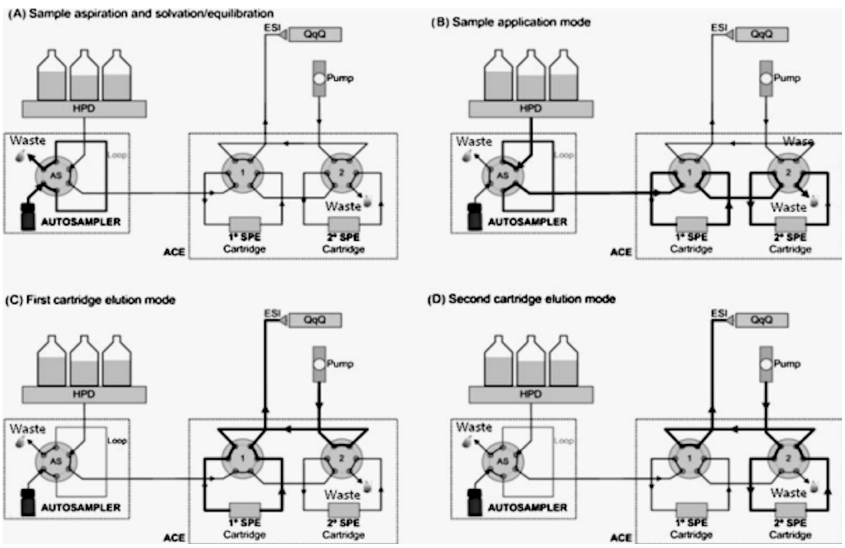
Supplementary Table 5. Comparison between results provided by DEQAS and the SPE–LC–MS/MS method for determination of 25(OH)D and 1,25$(OH)_2$D in human serum.

Total 25(OH)D					
Sample Nº	SPE–LC–MS/MS value (ng/mL)	LC–MS/MS methods mean value (ng/mL)	BIAS from LC–MS/MS method mean (%)	ALTM* (ng/mL)	BIAS from ALTM* (%)
451	34.8	32.3	7.7	32.4	7.4
452	119.8	116.9	2.5	114.9	4.3
453	71.6	66.5	7.7	60.7	18.0
454	49.4	53.5	-7.7	50.3	-1.8
455	60	55.5	8.1	55.5	8.1
456	75.4	77.1	-2.2	81.9	-7.9
457	46.4	42.3	9.7	42	10.5
458	70.6	78	-9.5	74.5	-5.2
459	90.1	98.7	-8.7	99.4	-9.4
460	56.5	54.2	4.2	55.3	2.2
Total 1,25$(OH)_2$D					
Sample Nº	SPE–LC–MS/MS value (pg/mL)	LC–MS/MS methods mean value (pg/mL)	BIAS from LC–MS/MS methods mean (%)	ALTM* (pg/mL)	BIAS from ALTM* (%)
341	139.3	125.5	11.0	133.8	4.1
342	84.8	76.1	11.4	80.5	5.3
343	110.1	100.4	9.7	114.3	-3.7
344	91.5	91.9	-0.4	87.1	5.1
345	92.1	84.5	9.0	103.4	-10.9
346	153	121.6	25.8	155.5	-1.6
347	115	96.3	19.4	109.7	4.8
348	89.4	81.9	9.2	93.5	-4.4
349	110	106.5	3.3	129.9	-15.3
350	82.9	72.2	14.8	76.9	7.8

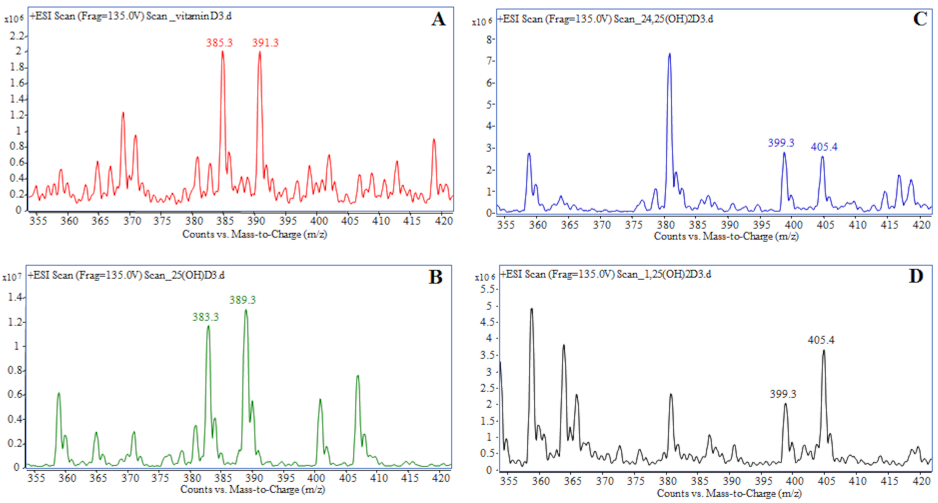
ALTM* = All-Laboratory Trimmed Mean.



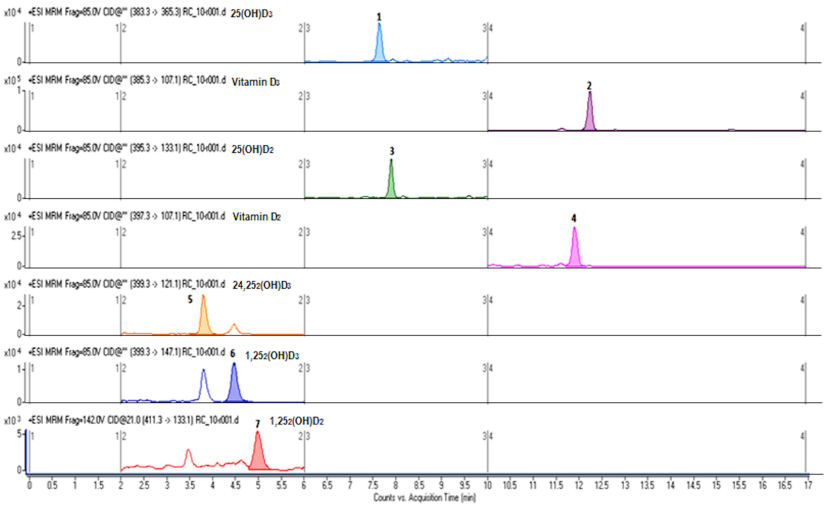
Supplementary Fig. 1.A. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method.



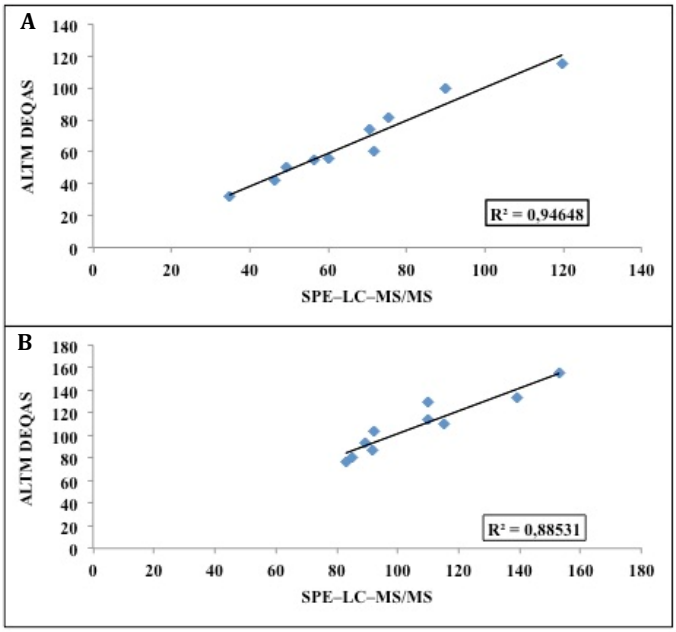
Supplementary Fig. 1.B. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method for estimation of the recovery factor. (A) Sample aspiration and solvation/equilibration. (B) Sample application mode. (C) First cartridge elution mode. (D) Second cartridge elution mode.



Supplementary Fig. 2. Mass spectrometry scans obtained from vitamin D_3 and its metabolites illustrating precursor ions for analytes and deuterated SIL-ISs.



Supplementary Fig. 3. SRM chromatograms obtained from serum spiked with100 ng/mL of vitamin D_2 and D_3, 50 ng/mL of monohydroxymetabolites and 10 ng/mL of dihydroxymetabolites.(1) 25(OH)D_3, (2) vitamin D_3, (3) 25(OH)D_2, (4) vitamin D_2, (5) 24,25$(OH)_2D_3$, (6) 1,25$(OH)_2D_3$ and (7) 1,25$(OH)_2D_2$.



Supplementary Fig. 4. Comparison between target concentrations provided by DEQAS and the results obtained by the SPE–LC–MS/M method. (A) total 25(OH)D and (B) total 1,25$(OH)_2$D.

Capítulo 2

Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D_3

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Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D_3

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Abstract

A method based on automated on-line solid phase extraction coupled to two-dimensional liquid chromatography with tandem mass spectrometry detection (SPE-2DLC–MS/MS) is here reported for vitamin D metabolite profiling in human serum with absolute quantification. Two-dimensional LC was configured with two complementary analytical columns, pentafluorophenyl (PFP) and C18 phases, for determination of 25 hydroxyvitamin D_3 epimers and the rest of bioactive metabolites of vitamin D (D_3 and D_2): 25-hydroxyvitamin D_2, 1,25-dihydroxy-vitamin D_3, 1,25-dihydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_3. Quantitative determination was supported on the use of a stable isotopic labeled internal standard for each analyte and the resulting method was validated by analysis of a standard reference material certified by the National Institute of Standards & Technology (NIST-972a) and 5 samples provided by the vitamin D External Quality Assurance Scheme (DEQAS). The limits of detection were between 9 and 90 pg/mL for the eight analytes, and precision, expressed as relative standard deviation, was lower than 11.6%. Two-dimensional LC has shown to be the key to discriminate

between 25 hydroxyvitamin D_3 epimers in a quantitative analysis also involving dihydroxyvitamin D metabolites.

Keywords: Vitamin D, Mono/dihydroxy metabolites, 3-epi-25(OH)D_3, Two-dimensional LC, Mass spectrometry, DEQAS, NIST

1. Introduction

A huge rise in the interest of the clinical effects of vitamin D and its metabolites has resulted in an increase in the development of new methods for their determination. In addition to the well known role of vitamin D to maintain calcium homeostasis and prevent rickets and osteomalacia [1], other diseases such as pathogenesis of autoimmune diseases, cardiovascular disorders, infectious diseases [2], even inhibition of progression of breast, colon or pancreas cancer [1] have been related to abnormal concentrations or ratios of vitamin D metabolites in humans. The levels of the target compounds are usually determined in serum or plasma, being the most frequently quantified analytes vitamin D_3, $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ [3,4]. Although the bioactive form of vitamin D_3 is the $1,25(OH)_2D_3$ metabolite, its concentration at the pg/mL level and possible cross-reactivity with $24,25(OH)_2D_3$ in most of the assays routinely used [2] have led to the use of the $25(OH)D_3$ metabolite, which is at the ng/mL level, as a biomarker of vitamin D status. Among the available methods to determine the concentration of $25(OH)D_3$, which range from immunoassays (RIA, ELISA, CPB) [5–8] to those based on chromatographic separation and determination by molecular UV–vis absorption or mass spectrometry (MS) [9], methods based on MS are at present the best as they do not suffer from limitations of specificity, cross-reactivity or low sensitivity. Therefore, MS methods are considered as the gold standard for analysis of endocrine hormones as they provide enough sensitivity for all vitamin D metabolites [2], which can be even improved by the use of automated solid phase extraction (SPE). This combination can give place to a clinical routine method that requires short analysis time (30 min) and low sample volume (250 μL) to determine seven analytes [9,10].

Recent studies have shown the presence of the C3-epimer form of $25(OH)D_3$ in human serum [11], thus revealing that vitamin D can also be metabolized through a parallel pathway [12,13], despite the enzyme responsible for 3-epimerization has

not been identified [13]. Also the subsequent step in the metabolism of the C3-epimer form [3-epi-25(OH)D_3] to yield 3-epi-1,25$(OH)_2D_3$ has been detected, and this dihydroxy form determined [12]. Bailey *et al.* have summarized some of the most important physiological functions of these metabolites [1], but more research is required to puzzle out the physiological function of C3-epimers of vitamin D [14]. It has been established that the 3-epi-25(OH)D_3 represents 60% of total 25(OH)D [15] in infants, and only 6.1% in adults [13]; therefore, quantification of 3-epi-25(OH)D_3 and 25(OH)D_3 might be an added value in a clinical routine method once the role of the epimer form is clarified. As both metabolites have identical elemental composition but different structure, they undergo identical selected reaction monitoring (SRM) transitions, thus leading to overestimation of 25(OH)D_3 concentration in most LC–MS/MS methods, which are unable to differentiate co-eluted epimers. Pentafluorophenyl (PFP) or CN-bonded chromatographic columns have been reported for separation of both analytes [1], being the former preferred by some authors as it provides better resolution [16].

The way to express the concentration of the C3-epimer in the LC–MS/MS methods published so far has been either as relative percentage considering as 100% the concentration of the two forms of the monohydroxymetabolite [15], or jointly with the 25(OH)D_3 form. Confirmation of the 3-epi-25(OH)D_3 peak by a stable isotopic labelled internal standard (SIL-IS), for example, the d_3-3-epi-25(OH)D_3, had not been carried out so far. According to the present information on this matter, a new analytical method has been developed for vitamin D metabolite profiling in human serum, including the 3-epi-25(OH)D_3 form. The method involved a special configuration based on two-dimensional liquid chromatography (2DLC) on-line coupled to solid-phase extraction and tandem mass spectrometry detection. Two complementary columns, a pentafluorophenyl (PFP) column and a C18 column, were used for separation of the two 25(OH)D_3 epimers without affecting the sensitivity and resolution demanded for determination of the other vitamin D metabolites.

2. Experimental

2.1. Chemicals and reagents

The monohydroxymetabolites 25(OH)D_2, 25(OH)D_3 and 3-epi-25(OH)D_3, dihydroxymetabolites 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$, and vitamins D_2 and D_3 were from Sigma–Aldrich(St. Louis, MO, USA). Stable isotopic standards 1,25$(OH)_2D_3$–d_6, 24,25$(OH)_2D_3$–d_6, and 25(OH)D_3–d_6 were provided by A. Mouriño (Department of Organic Chemistry, University of Santiago, Santiago de Compostela, Spain) and M.A. Maestro (Department of Fundamental Chemistry, University of La Coruña, La Coruña, Spain), while 3-epi-25(OH)D_3-d_3, 25(OH)D_2-d_3, vitamin D_2-d_3, and vitamin D_3-d_6 were from Sigma–Aldrich. One multistandard working solution was prepared with the target analytes at different concentrations: 100 ng/mL for dihydroxymetabolites 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$; 1 µg/mL for 24,25$(OH)_2D_3$; 5 µg/mL for 25(OH)D_3, 3-epi-25(OH)D_3 and 25(OH)D_2; and 10 µg/mL for vitamin D_2 and vitamin D_3. Other solution was prepared with all isotopic standards –7.5 ng/mL for 1,25$(OH)_2D_3$-d_6 and 125 ng/mL 24,25$(OH)_2D_3$-d_6; 625 ng/mL for 25(OH)D_3-d_6, 3-epi-25(OH)D_3-d_3, 25(OH)D_2-d_3, vitamin D_2-d_3 and vitamin D_3-d_6.

LC–MS grade reagents and solvents were used in this research. Ammonium formate from Sigma–Aldrich and acetonitrile, formic acid and methanol from Scharlab (Barcelona, Spain) were used for preparation of chromatographic mobile phases and solutions used for SPE.

2.2. Instruments and apparatus

The analyses were performed by 2DLC in reversed-phase mode followed by electrospray ionization in positive mode (ESI +) and MS/MS detection in Selected Reaction Monitoring (SRM) acquisition mode. Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer. The analytical columns

were Pursuit PFP (3 μm particle size, 100×4.6 mm i.d.) and Poroshell 120 EC-C18 (2.7 μm particle size, 50×4.6 mm i.d.), both from Agilent. A guard column (2.7 m particle size, 5.0×2.1 mm i.d.), also from Agilent, was used to preserve the integrity of the analytical columns. The data were processed using MassHunter Workstation Software (V-B.05) for qualitative and quantitative analysis. On-line SPE was performed by a Symbiosis system (Spark Holland, Emmen, The Netherlands). This commercial equipment comprises a unit for automatic cartridge exchange (ACE), an autosampler (Reliance) furnished with a 0.2 mL sample loop and two high-pressure syringe dispensers (HPDs) for SPE solvent delivery. Peek tubing of 0.25 mm i.d. (VICI, Houston, Texas,USA) was used to connect all valves of the Symbiosis unit and LC–MS/MS modules. Peek tubing of 1.0 mm i.d. and 130 cm length, about 1 mL volume, was used to connect the Reliance to the ACE unit for mixing the serum sample and loading solution. A 10×2 mm cartridge packed with Hysphere C8 (Spark Holland) as sorbent material was used for SPE.

2.3. Sampling, sample collection and storage

All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki (2004), which were supervised by the ethical review board of Reina Sofia University Hospital (Córdoba, Spain) that approved the experiments. Venous blood from each individual was collected into a Vacutainer® (Becton Dickinson) plastic serum tube with spray-coated silica and a polymer gel to favor serum separation. All collection tubes were processed by centrifugation at 2000×*g*. After that, the samples were placed in plastic ware tubes and stored at −80 ºC until analysis. Five human serum samples were provided by DEQAS for analysis of 1,25(OH)2D [$1,25(OH)_2D_3$+ $1,25(OH)_2D_2$] using the proposed method. The samples were prepared from individual blood donations and were sterilized by filtration through 0.2 μm microbiological grade filters by the Oncology/Endocrinology Laboratory of Charing Cross Hospital (Fulham Palace Rd, London W6 8RF, UK). A standard

reference material (NIST-972a) consisting of four human serum samples with certified concentrations of 25(OH)D_3, 3-epi-25(OH)D_3 and 25(OH)D_2 by NIST were tested with the proposed 2DLC approach for validation of the method.

2.4. Proposed procedure

A volume of 240 µL of serum in an amber glass vial was spiked with 10 µL of the deuterated working solution-final concentration in serum: 25 ng/mL of 25(OH)D_3-d_6, 3–epi–25(OH)D_3–d_3, 25(OH)D_2-d_6, vitamin D_3-d_3 and vitamin D_2-d_6, 5 ng/mL of 24,25$(OH)_2D_3$-d_6 and 0.3 ng/mL of 1,25$(OH)_2D_3$-d_6-, shaken and located in the autosampler. Figure 1 shows the analytical platform used for determination of vitamin D and its metabolites.

The sample loop was filled with 0.2 mL of filtered serum from the sample vial, which was refrigerated at 6 ºC in the Reliance unit. The serum was subjected to the sequence of automatic operations described in Supplementary Table 2 that were optimized in previous publications [3,9]. Elution from the SPE cartridge was carried out with the mobile phase for 6 min to the PFP analytical column, which was initially on-line connected to the C18 column. At min 13 of the chromatographic program (when the dihydroxymetabolites reach the C18 column) valve-2 switches and the eluting solution from the PFP column is directly led to the QqQ detector to complete the chromatographic method. Thus, the monohydroxy-metabolites do not pass through the C18 column. At min 18 the valve-2 switches again to the initial configuration with the on-line connection of the two columns for separation of vitamin D forms in the C18 column.

The mobile phases were water (phase A) and methanol (phase B) both containing 5 mM ammonium formate as ionization agent. Initially, the mobile phase was 80:20 B/A at a flow rate of 0.3 mL/min, and the first gradient was from 80% to 85% phase B in 5 min and10 min to reach 90% phase B, followed by a ramp from 90% to 92% phase B in 1 min. Then, 4 min at a 5 mL/min to rise 100% phase B.

The final gradient conditions were maintained for 8 min until the end of the chromatographic step. The total analysis time was 28 min, and 10 additional min were required for re-establishing and equilibrating the initial conditions. The column temperature was constant at 10 ºC during the analysis. A final step in the Symbiosis system was to purge tubing and cartridge for reuse. The chromatographic–detection step of a sample and the SPE step of the next sample overlapped, thus improving the analysis frequency.

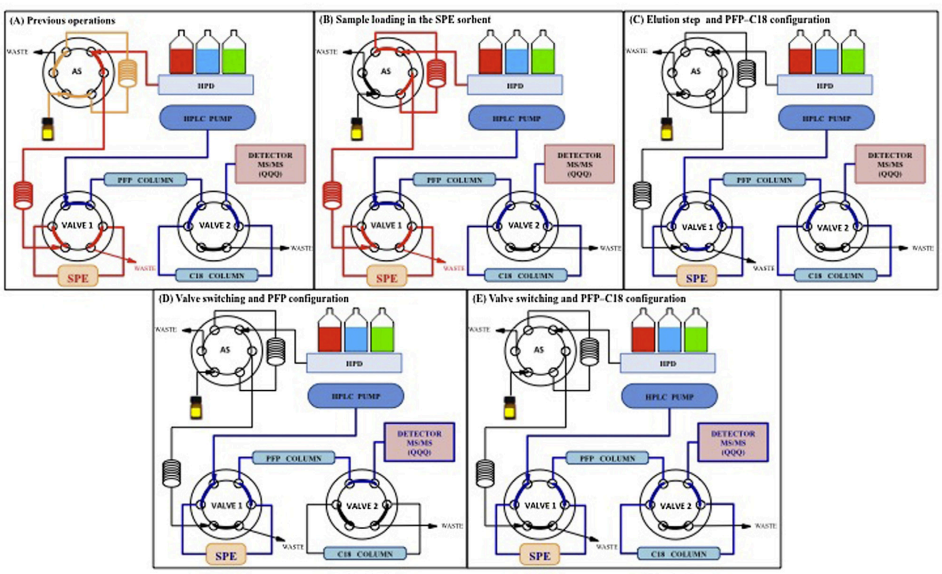

Fig. 1. Scheme of the SPE-2DLC–MS/MS approach used in the proposed method. (A) Sample aspiration and equilibration/solvation of the SPE sorbent. (B) Sample loading inthe SPE sorbent. (C) Elution of the target analytes from the SPE sorbent to the PFP-C18 configuration. (D) Valve switching for determination of monohydroxymetabolites afterPFP separation. (E) Valve switching for separation of dihydroxymetabolites and vitamin D analytes in the C18 column.

The eluate from the chromatographic columns was monitored by MS/MS in SRM, the information of which appears in Table 1. The flow and temperature of the drying gas (N_2) were 9 L/min and 350 ºC, respectively. The nebulizer pressure was 50 psi, and the capillary voltage in positive mode was 4750 V.

Table 1. Optimization of the MS/MS SRM method for quantitative and confirmatory analysis of vitamin D metabolites and internal standards.

Analyte	Precursor ion (*m/z*)	Adducts	Quantitation transition	Collision energy (V)	Retention time (min)	Internal standard	Precursor ion (*m/z*)	Quantitation transition	Collision energy (V)
$24,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	121.1	30	19.4	$24,25(OH)_2D_3$-d_6	405.4	121.1	20
$1,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	227.1	20	20.3	$1,25(OH)_2D_3$-d_6	405.4	227.1	20
$1,25(OH)_2D_2$	411.3	$[M+H-(H_2O)]^+$	133.1	20	20.7				
$25(OH)D_3$	383.3	$[M+H-(H_2O)]^+$	107.1	30	13.5	$25(OH)D_3$-d_6	389.3	107.1	25
3epi-$25(OH)D_3$					14	3epi-$25(OH)D_3$-d_3	386.4	107.1	30
$25(OH)D_2$	395.3	$[M+H-(H_2O)]^+$	107.1	30	13.9	$25(OH)D_2$-d_3	398.4	107.1	30
Vit D_2	397.3	$[M+H]^+$	379.2	5	26.7	Vit D_2-d_3	400.4	382.1	10
Vit D_3	385.3	$[M+H]^+$	107.1	30	26.9	Vit D_3-d_3	385.3	107.1	25

2.5. Method validation

Three serum pools were prepared by spiking them with the target analytes at low, intermediate and high concentrations according to the physiological levels described in the literature [17] (see the concentrations used for preparation of each pool in Supplementary Table 1). The pools were used for evaluation of analytical features such as precision and recovery factor of the SPE step.

A calibration model was developed for each analyte using a serum pool, aliquots of which were spiked with different concentrations of the target analytes and with a constant concentration of the deuterated standards. Also, a blank serum spiked

only with deuterated standards was prepared to correct the endogenous concentration of the target analytes in the serum pool.

2.6. Data treatment

Quantification was carried out using the ratio between the peak area of each analyte and that of the corresponding SIL-IS. Quantitative analysis of $1,25(OH)_2D_2$ was carried out by using $1,25(OH)_2D_3$ as IS due to their structural similarity.

3. Results and discussion

3.1. Comparison of PFP and C18 columns

The PFP and C18 columns were compared in terms of capacity to separate the vitamin D metabolites under study, the 3-epi-$25(OH)D_3$ form included. The sensitivity, defined by the limit of quantification (LOQ), for determination of $1,25(OH)_2D_3$ was a crucial factor taking into account that this metabolite is present at the lowest concentration range of vitamin D metabolites in human serum. With comparison purposes, a human serum sample pool spiked with the target analytes at intermediate concentrations (Supplementary Table 1) and with SIL-ISs was analyzed in the SPE–LC–MS/MS arrangement using each column separately. The reversed phase gradient described above for two-dimensional chromatography was used for these tests. Figure 2 shows the chromatograms provided by each column. As can be seen, the PFP column, with a separation mechanism based on interaction of hydroxyl groups with the sorbent, allowed separation of the monohydroxy-metabolites with special emphasis on 3-epi-$25(OH)D_3$, but with very low sensitivity for $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ forms (essential component of the metabolism profile of vitamin D).

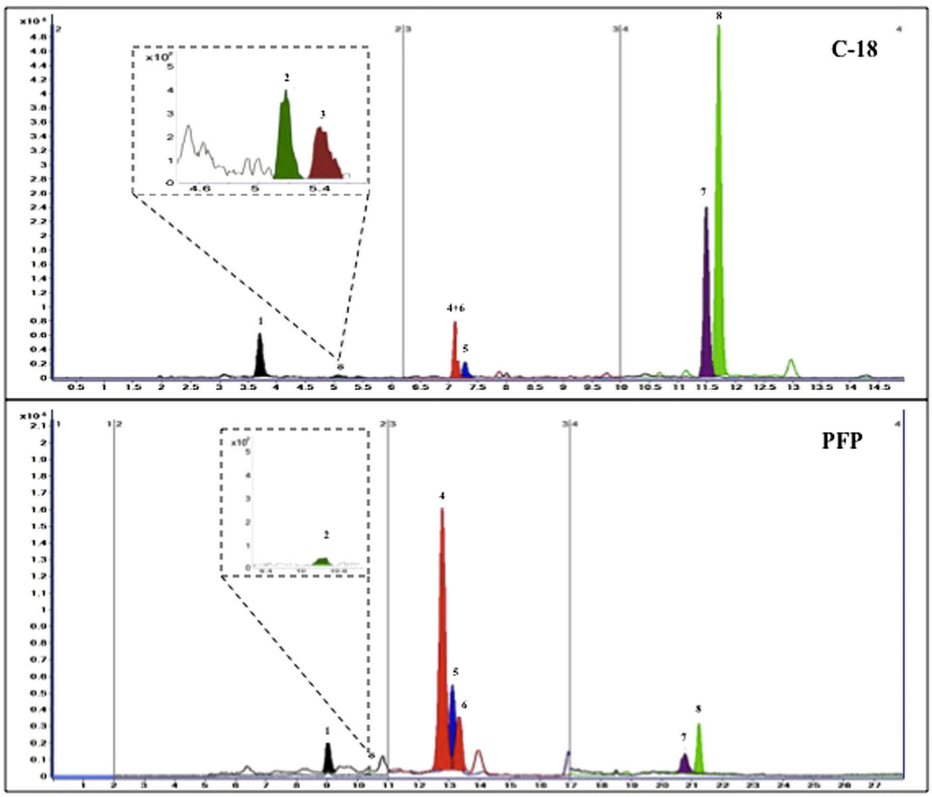

Fig. 2. MRM chromatograms obtained with the two different columns (C18 and PFP) after analysis of serum spiked with 100 ng/mL of vitamin D_2 and D_3, 50 ng/mL of monohydroxy-metabolites and 10 ng/mL of dihydroxy metabolites. (1) $24,25(OH)_2D_3$, (2) $1,25(OH)_2D_3$ and (3) $1,25(OH)_2D_2$, (4) $25(OH)D_3$, (5) $25(OH)D_2$, (6) 3-epi-$25(OH)D_3$,(7) vitamin D_2, and (8) vitamin D_3.

Thus, the quantification of these two dihydroxymetabolites, usually at pg/mL, was not possible, in contrast to $24,25(OH)_2D_3$ that is normally present at concentrations within ng/mL. The chromatogram provided by the C18 column

(Figure 2) shows that this sorbent enabled to achieve the demanded sensitivity for determination of 1,25$(OH)_2D_3$, as previously reported [3,9], but it was notable to discriminate the two C-3 epimer forms of 25$(OH)D_3$. It is also worth mentioning that, although the sensitivity for vitamin D_2 and D_3, the less polar compounds, is not critical, it was considerably enhanced with the C18 column as compared to the PFP column. The differences in sensitivity can be explained by the ionization efficiency attained in both methods despite the same chromatographic gradient was used. As can be seen, vitamin D_2 and D_3 were eluted in 12 min with the C18 based method, while 22 min were required in the PFP approach. Therefore, ionization of the analytes in the C18 method was carried out under a more aqueous medium than in the PFP method. With these premises, a two-dimensional chromatographic separation combining both columns was assayed to take benefits from the high sensitivity attained with C18 chromatographic separation and from resolution of PFP sorbent with discrimination capability for 25$(OH)D_3$ epimers.

3.2. Two-dimensional chromatography configuration

According to the results presented above, a two-dimensional configuration was adopted for combined analysis of monohydroxymetabolites epimers and dihydroxy metabolites. The PFP column was mandatory for separation of epimers, while the C18 sorbent allowed determining the dihydroxymetabolites with high sensitivity. For this reason, the PFP column was connected to the C18 column by a switching valve (see Figure 1) in a PFP-C18 configuration. This valve enables to lead the PFP eluate either directly to the mass spectrometer or to the C18 column for 2DLC separation prior to detection. This switching valve was automatically programmed according to the elution order of the three groups of analytes: dihydroxy vitamin D metabolites, monohydroxy vitamin D metabolites and vitamin D forms. Despite initially the valve was set for 2DLC separation, it was switched at 13 min to avoid interaction of monohydroxy metabolites with the C18 column since resolution in the separation of 25$(OH)D_3$ epimers would be affected. Then, at

18 min the valve was switched again to the initial configuration to allow the passage of vitamin D forms through the C18 column. Therefore, the monohydroxy metabolites interacted only with the PFP column, while the rest of the analytes interacted with both columns since the C18 separation increased the sensitivity. Figure 3 shows the chromatogram obtained using this configuration, in which the monohydroxymetabolites appear separated, followed by the dihydroxymetabolites and, finally, vitamins D_3 and D_2.

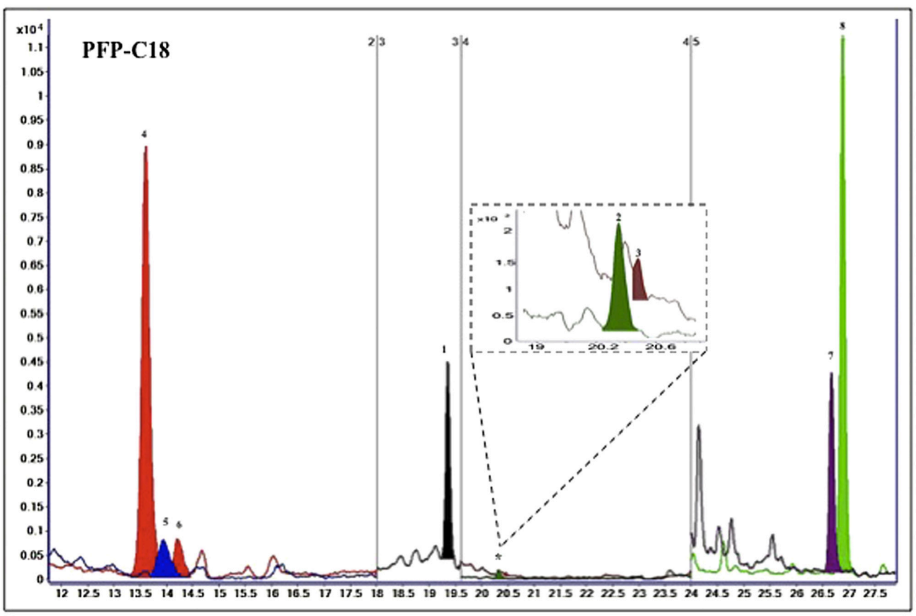

Fig. 3. MRM chromatograms obtained by the two-dimensional chromatography configuration (PFP-C18) from serum spiked with 100 ng/mL of vitamin D_2 and D_3, 50 ng/mL of monohydroxy metabolites and 10 ng/mL of dihydroxy metabolites. (1) $24,25(OH)_2D_3$, (2) $1,25(OH)_2D_3$ and (3) $1,25(OH)_2D_2$, (4) $25(OH)D_3$, (5) $25(OH)D_2$, (6) 3-epi-$25(OH)D_3$,(7) vitamin D_2, and (8) vitamin D_3.

As can be seen, retention of the dihydroxymetabolites in the C18 column changed the elution order, being the monohydroxy metabolites the first group of

compounds to be detected as they were directly eluted from the PFP column to the QqQ analyzer. The reverse configuration, C18-PFP column did not provide successful results since resolution in the separation of 25(OH)D_3 epimers was critically affected (Supplementary Fig. 1). Modifications of the chromatographic gradient improved the resolution of epimers separation, but also affected critically to the separation of dihydroxymetabolites and, for this reason, this option was discarded.

3.3. Analytical characteristics of the method

3.3.1. Sensitivity

Calibration models were prepared using pools of human serum from donors, which were spiked with vitamin D and metabolites at different concentrations, and also with the SIL-IS for each compound. Table 2 lists the principal analytical features of the calibration models. The limits of detection (LOD) and LOQ for each metabolite were calculated as the concentration providing signals three and ten times, respectively, higher than the background noise measured at retention time close to each chromatographic signal. All calibration models provided regression coefficients above 0.99. As shown in Table 2, the method is endowed with appropriate sensitivity to quantify all the analytes at their normal levels in human serum [17], ranging from ng/mL to pg/mL. It is worth mentioning that the sensitivity for 3-epi-25(OH)D_3 was quite similar to that obtained for 25(OH)D_3, which means that this analytical feature was not affected by epimerization. Additionally, the sensitivity provided by this 2DLC configuration was quite similar to that reported previously with the same sort but using C18 chromatographic separation [3,9].

Table 2. Analytical characteristics of the method.

Analyte	LOD (pg/mL)	LOQ (ng/mL)	Calibration range	Equation	Regressio coefficient	Within-day variability (%)	Between-days variability (%)
$24,25(OH)_2D_3$	90	0.3	0.3 ng/mL –50 ng/mL	y = 0.39x + 0.042	0.994	3.2	8.2
$1,25(OH)_2D_3$	9	0.03	30 pg/mL – 2.5 ng/mL	y = 0.0021x + 0.454	0.995	3.9	7.8
$1,25(OH)_2D_2$	15	0.05	50 pg/mL– 50 ng/mL	y = 2.065x – 0.425	0.996	6.4	11.3
$25(OH)D_3$	75	0.25	0.25 ng/mL –250 ng/mL	y = 0.514x – 0.427	0.997	7.2	9.9
$3epi\text{-}25(OH)D_3$	90	0.3	0.3 ng/mL –250 ng/mL	y = 0.240x + 0.241	0.996	4.1	4.6
$25(OH)D_2$	75	0.25	0.25 ng/mL –250 ng mL	y = 0.622x – 0.548	0.996	4.4	5.3
Vit D_2	30	0.1	0.1 ng/mL –250 ng/mL	y = 0.171x – 0.473	0.990	6.0	8.9
Vit D_3	30	0.1	0.1 ng/mL–250 ng/mL	y = 1.276x –1.5581	0.996	2.1	11.6

3.3.2. Precision

The precision of the method was evaluated by calculation of the within-day and between-days variability both expressed as relative standard deviation (RSD). For this purpose, a single experimental set-up by duplicate analysis each day (for 7 days) was carried out with the serum pool spiked with the target analytes at intermediate concentrations (Supplementary Table 1) according to the physiological levels described in the literature [17]. The obtained results, listed in Table 2, shows that precision, expressed as RSD, was below 11.6%, and the within-day variability was below 7.2% for all analytes. The precision estimation for 3-epi-$25(OH)D_3$ was slightly lower than the values reported for the $25(OH)D_3$ form and, in general terms, the precision was not affected by application of a 2DLC separation as compared to the previously reported method that was not targeted at epimeric discrimination of $25(OH)D_3$ [3,9].

3.3.3. Recovery factor

The recovery factor was calculated using the three pools of human serum spiked at low, intermediate and high concentrations of the target analytes (Supplementary Table 1) and using the "two-cartridges configuration" of the SPE workstation

(illustrated in Supplementary Fig. 2) [3,9]. This configuration allows checking if the target analytes are quantitatively retained in one SPE cartridge at the typical concentration range. The recovery factor was calculated as amount of analyte retained in cartridge 1/[amount of analyte in cartridge 1 + amount of analyte in cartridge 2]. The values obtained for the three spiked levels allowed obtaining an average recovery factor for each analyte, as shows Table 3. The recovery factors were around 100% for dihydroxymetabolites, which is crucial for their quantification in serum, while the monohydroxymetabolites, including the 3-epimer, were above 95.2%. On the other hand, the recovery factor decreased considerably up to 59.1 and 60.2% for vitamin D_2 and D_3, respectively, which could be explained by a low extraction efficiency of the C18 sorbent for the two less polar forms. Nevertheless, these two analytes are present at high physiological levels as compared to the vitamin D metabolites and, therefore, quantitative extraction is not a key requirement.

Table 3. Estimation of recovery for the SPE step and accuracy of the method for quantitative analysis of each analyte.

Analyte	Recovery factor (%)	Accuracy (%)
$24,25(OH)_2D_3$	100	89.3
$1,25(OH)_2D_3$	100	95.9
$1,25(OH)_2D_2$	99.8	90.9
$25(OH)D_3$	98.8	96.1
$3epi\text{-}25(OH)D_3$	95.2	95.5
$25(OH)D_2$	97.2	95.4
Vit D_2	59.1	83.3
Vit D_3	60.2	93.6

3.3.4. Accuracy of the method

The accuracy of the method and potential matrix effects were studied for each analyte by using the three serum pools spiked at three different concentration levels as previously described (Supplementary Table 1). The accuracy was calculated by the ratio [measured concentration–endogenous concentration]/spiked concentration. According to this equation, the monohydroxylated metabolites reported values of 96.1 and 95.4% for 25-hydroxy vitamin D_3 and D_2, respectively, and 95.5% for the C3-epimer. The sensitivity of the method for these analytes is appropriate for their quantification in human serum. The average accuracy for the dihydroxylated metabolites ranged from 89.3 to 95.9%, which are within the precision range previously estimated. Finally, vitamins D_2 and D_3 reported average accuracy values of 83.3 and 93.6%, respectively, which means that despite the low recovery factor provided by the two vitamin D forms, they could be accurately quantified.

3.4. External validation of the proposed method by analysis of NIST standard reference material

The method was externally validated by analysis of the standard reference material NIST-972a, which consists of serum samples with concentrations of 3-epi-25(OH)D_3, 25(OH)D_3 and 25(OH)D_2 certified by NIST. These serum samples encompassed a wide range of concentrations of the three target monohydroxymetabolites, which were within the normal values described in the literature [17]. The concentration levels ranged from 0 to 29.4 ng/mL of 3-epi-25(OH)D_3, from 18.1 to 29.4 ng/mL of 25(OH)D_3 and from 0 to 13.3 ng/mL of 25(OH)D_2. The obtained results after in triplicate analysis of each sample, and the certified concentrations are listed in Table 4, with the bias for each measurement expressed as percentage. As can be seen, the percentage of bias by comparison with the reference values ranged from 2.0 to 8.5% that emphasizes the accuracy of

the proposed approach for analysis of monohydroxymetabolites with discrimination of 25(OH)D_3 epimers.

Table 4. Comparison between results provided by DEQAS and the SPE–LC–MS/MS method for determination of 25(OH)D and 1,25$(OH)_2$D in human serum.

Total 25(OH)D					
Sample Nº	**SPE–LC–MS/MS value (ng/mL)**	**LC–MS/MS methods mean value (ng/mL)**	**BIAS from LC–MS/MS method mean (%)**	**ALTM* (ng/mL)**	**BIAS from ALTM* (%)**
476	92.6	101.1	8.3	99.7	7.0
477	43.2	45.3	4.4	42.8	-1.1
478	67.9	74.6	8.9	74.1	8.3
479	30.0	32.4	7.0	30.8	2.4
480	97.6	103.6	10.3	97.7	4.9
Total 1,25$(OH)_2$D					
Sample Nº	**SPE–LC–MS/MS value (pg/mL)**	**LC–MS/MS methods mean value (pg/mL)**	**BIAS from LC–MS/MS methods mean (%)**	**ALTM* (pg/mL)**	**BIAS from ALTM* (%)**
366	85.2	100.8	-15.5	110.3	-22.7
367	72.9	102.1	-28.6	105.2	-30.7
368	84.5	79.1	6.8	85.5	-1.2
369	116.6	112.5	3.6	128.7	-9.4
370	98.0	130.7	-25.0	147.3	-33.5

Since the used standard reference material does not provide certified concentrations for the dihydroxymetabolites, five serum samples provided by DEQAS (code number 366-370) were analyzed in triplicate with the proposed approach to determine the total concentration of 1,25$(OH)_2$D, which is the reference value used by the DEQAS program. The reference values were calculated as the average concentration according to values reported by all laboratories using LC–MS/MS methods. Table 5 lists the reference concentrations for the five serum samples, the obtained results and the bias percentage. As can be seen, the percentage of bias ranged from 3.6 to 28.6%. These deviations are within the limits established by DEQAS, set at ±30% in this case taking into account that this metabolite is normally in serum at the pg/mL level.

Table 5. Comparison between results provided by NIST and the SPE–LC–MS/MS method

Level	Analytes	SPE–LC–MS/MS value (ng/mL)	Certified NIST value (ng/mL)	BIAS (%)
1	$25(OH)D_3$	28.2	28.8	2.0
	3-epi-$25(OH)D_3$	1,75	1.84	4.7
2	$25(OH)D_2$	0.74	0.81	7.8
	$25(OH)D_3$	16.5	18.1	8.5
	3-epi-$25(OH)D_3$	1.20	1.29	6.4
3	$25(OH)D_2$	14.2	13.3	-6.7
	$25(OH)D_3$	18.6	19.8	5.7
4	$25(OH)D_3$	27.3	29.4	7.2
	3-epi-$25(OH)D_3$	27.2	29.4	-8.1

3.5. Comparison of the proposed method with previous methods dealing with the analysis of 3-epi-25(OH)D₃

Comparison between a new method and those existing in the literature requires discussion of the improvements attained with the proposed approach. As the most remarkable aspects of the proposed method is the inclusion of 3-epi-$25(OH)D_3$, the comparison is exclusively focused on methods involving quantitative analysis of the epimer forms. It has been recently when methods for determination of vitamin D metabolites have included 3-epi-$25(OH)D_3$ among the target analytes. The methods in which a single chromatographic column is used are able to determine only monohydroxymetabolites [5,6,8,15,16,18,19], ignoring the rest of interesting biological active metabolites of vitamin D such as $24,25(OH)_2D_3$, $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, and even vitamin D_3 and D_2. Only Beacher *et al.* included $24,25(OH)_2D_3$ among their target analytes [19]. A method involving two chromatographic columns was reported by Shah *et al.* [20], with a total of eight analytes (vitamin D and metabolites). The method does not include a key dihydroxy-

metabolite, 24,25(OH)$_2$D$_3$, known as the excretion metabolite of vitamin D. The method here proposed includes 24,25(OH)$_2$D$_3$. In addition, as compared with that of Shah *et al.*, our method takes advantage of the amount of sample per analysis: 0.25 mL versus 1.0 mL required by Shah *et al.* These authors also used manual liquid–liquid extraction as sample preparation step, which requires longer time than the fully automated SPE sample preparation of the present method and more user involvement. In other way, the method from Shah *et al.* only use stanozolol- d$_3$ as internal standard, while this method uses the SIL-IS for each analyte. Finally, the levels of sensitivity are similar in both methods.

4. Conclusions

The proposed method, based on 2DLC separation, allows programming sequential elution of vitamins D$_2$ and D$_3$ and their metabolites prior to reaching the mass detector. The nature of the columns allows separation of eight analytes, and the sensitivity of the mass detector made possible their quantification at the levels they typically exist in human serum (ranging from μg/mL to pg/mL) using a small sample volume (250 μL). The method presents less sample preparation than one-column LC methods and has similar levels of sensitivity. The method has been characterized and validated by application to a standard reference material certified by NIST for monohydroxymetabolites including the 3-epimer of 25(OH)D$_3$. Additionally, five samples provided by DEQAS enabled to check the usefulness of the method for determination of 1,25(OH)2D.

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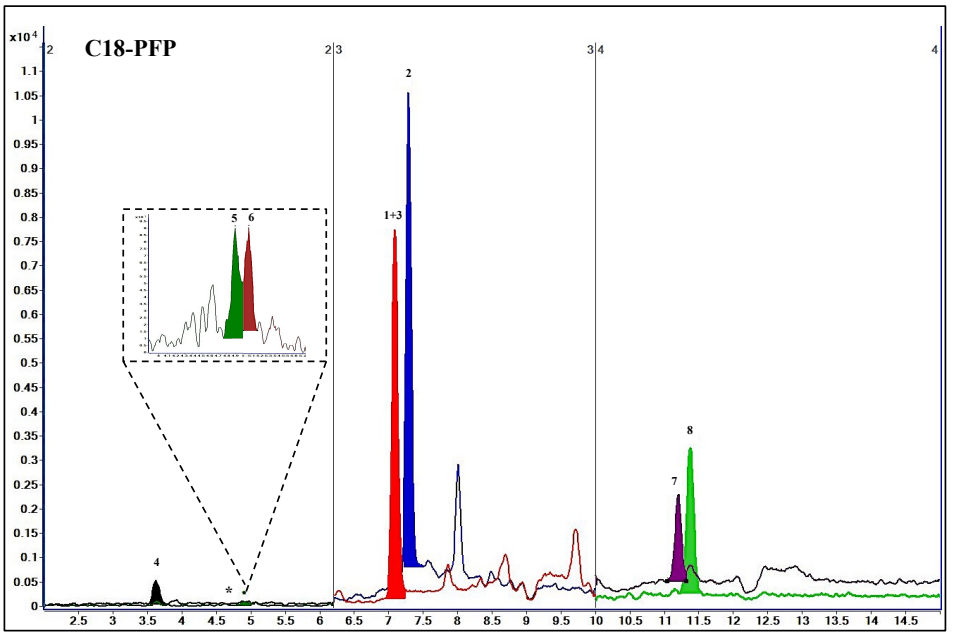
Supplementary Table 1. Concentrations of the target analytes spiked in human serum for estimation of the recovery factor and the accuracy study.

Analyte	Low level (ng/mL)	Intermediate level (ng/mL)	High level (ng/mL)
$1,25(OH)_2D_3$	0.05	0.1	0.2
$1,25(OH)_2D_2$	3	10	20
$24,25(OH)_2D_3$			
$25(OH)D_3$	15	50	100
$25(OH)D_2$			
Vit D_2	15	50	100
Vit D_3			

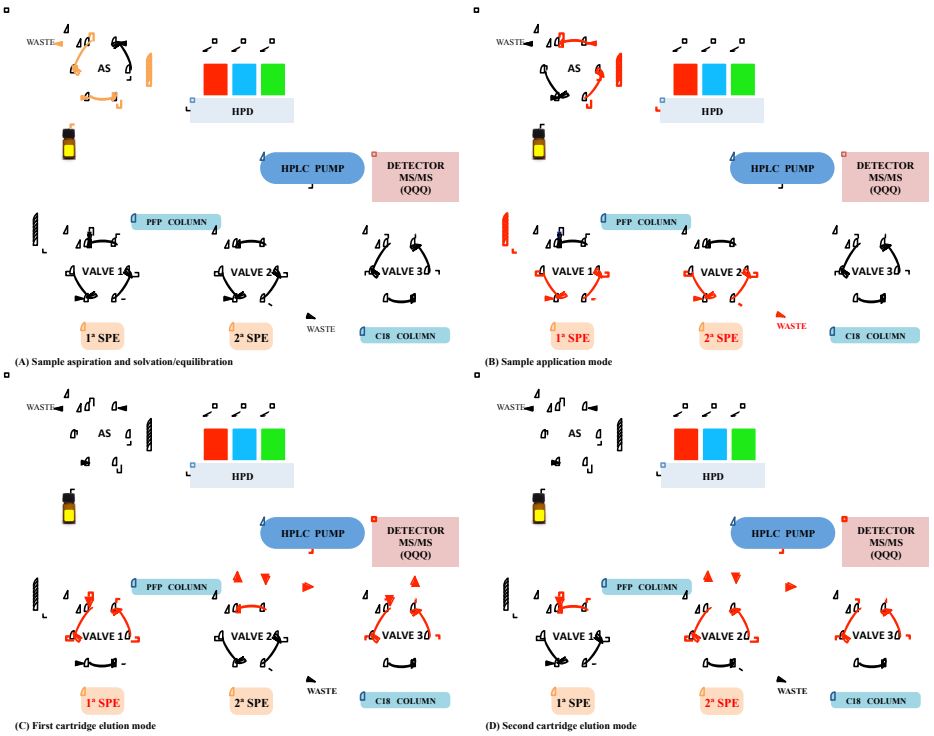
Supplementary Table 2. Steps programmed in the SPE protocol.

Step	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				
Start autosampler				Load sample
Solvation 1	10	2	Methanol	
Solvation 2	5	4	Methanol	
Equilibration	3	4	25% ACN–0.7% F*	
Equilibration	3	4	25% ACN–0.7% F*	
Sample application	3	4	25% ACN–0.7% F*	
Wash cartridge	3	0.5	30% ACN	
Elution			Mobile phase	6 min
Purge 1	5	5	Methanol	
Purge 2	5	5	30% ACN	
Purge 3	5	5	Water	
Purge 4	5	5	30% ACN	
Purge 5	5	5	Water	

*Formic acid



Supplementary Fig. 1. MRM chromatograms obtained with the two different columns (C18 and PFP) after analysis of serum spiked with 100 ng/mL of vitamin D2 and D3, 50 ng/mL of monohydroxymetabolites and 10 ng/mL of dihydroxymetabolites. (1) 24,25(OH)2D3, (2) 1,25(OH)2D3 and (3) 1,25(OH)2D2, (4) 25(OH)D3, (5) 25(OH)D2, (6) vitamin D2, (7) vitamin D3 and (8) 3-epi-25(OH)D3.



Supplementary Fig. 2. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method for estimation of the recovery factor. (A) Sample aspiration and solvation/equilibration. (B) Sample application mode. (C) First cartridge elution mode. (D) Second cartridge elution mode.

Estudios sobre la estabilidad y la preparación de la muestra para la determinación de vitamina D y sus metabolitos

Studies on stability and sample preparation for determination of vitamin D and its metabolites

SECTION II

La Sección II de esta Memoria de Tesis abarca la investigación llevada a cabo para optimizar las primeras etapas del método global, que es el objetivo general de la Tesis.

Un primer estudio se dedicó a la recogida y pretratamiento de la muestra, que se recoge en el Capítulo 3. Los diferentes recubrimientos internos de los tubos de recogida se estudiaron en función del analito finalmente determinado —principalmente 25(OH)D y 1,25$(OH)_2$D—, comparando así entre tipos de muestra aislada: suero o plasma. La selección del pretratamiento de la muestra implicó desproteinización y SPE, de las que esta última proporcionó los mejores valores de limpieza y de preconcentración. La selección de la muestra derivada de la de sangre total puso de manifiesto que el plasma proporcionó igualmente mayor intensidad de señal para los analitos, pero el suero dio lugar a una menor contribución del ruido de fondo que facilito la cuantificación de los analitos menos concentrados.

La estabilidad de la muestra es un aspecto clave que había sido pobremente estudiado hasta la fecha en relación con la vitamina D y sus metabolitos. Éste fue el objeto del Capítulo 4 de esta Memoria, que abarca un estudio de dos meses sobre la estabilidad de todos los analitos implicados en el método desarrollado. La influencia de la temperatura y del número de ciclos de congelación/descongelación durante el almacenaje de cada analito en las muestras de suero permitió establecer las mejores condiciones para su conservación. Un estudio adicional sobre la estabilidad de las muestras de suero liofilizadas puso de manifiesto que la liofilización es una alternativa de conservación adecuada de estas muestras.

Section II of this PhD-Book encompasses the research devoted to optimizing the first steps of the method, overall objective of the Thesis.

A first study was devoted to sample collection and sample pretreatment, constituting Chapter 3. The different coatings of the collection tubes were assayed as a function of the target analyte —mainly 25(OH)D and $1,25(OH)_2D$— to be finally determined, thus also comparing between the types of isolated samples: serum or plasma. Selection of sample pretreatment involved deproteination and SPE, with the latter providing better clean-up and preconcentration effects. Selection of the blood-derived sample showed that plasma provided a bit higher intensity signals from the target analytes, but serum gave place to a lower background contribution that facilitated quantification concentrated analytes.

Sample stability is a key aspect that had been poorly studied so far in dealing with vitamin D and its metabolites. For this reason, this was the subject of Chapter 4, which encompasses a two-month stability study of all the analytes involved in the developed method. The influence of temperature in sample-serum storage and the number of freeze/thaw cycles on each analyte were thus known and the best conditions for future storage established. An additional study on the stability of serum lyophilized samples showed this pretreatment as a suited alternative for sample conservation.

Capítulo 3

Study of blood collection and sample preparation for analysis of vitamin D and its metabolites by liquid chromatography–tandem mass spectrometry

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Study of blood collection and sample preparation for analysis of vitamin D and its metabolites by liquid chromatography–tandem mass spectrometry

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Abstract

The analysis of vitamin D_3 status, with special emphasis on 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3, is gaining interest in clinical studies due to the classical and non-classical effects attributed to this prohormone. In this research, the influence of the two steps preceding determination (*viz.*, sample collection and preparation) on the quantitative analysis of vitamin D_3 and its more important metabolites has been studied. Two preparation approaches, deproteination and solid phase extraction (SPE), have been evaluated in terms of sensitivity to decide their application, thus establishing that detection of 1,25-dihydroxyvitamin D_3 cannot be addressed by protein precipitation. Concerning sample collection, serum and plasma reported high accuracy (above 83.3%) for vitamin D_3 and metabolites, while precision, expressed as relative standard deviation, was below 12.9% for all analytes in both samples. Statistical analysis revealed that serum and plasma provided similar physiological levels for vitamin D_3, 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3, while significantly different levels were obtained for

1,25-dihydroxyvitamin D_3, always higher in plasma than in serum. Sample collection and treatment have proved to be significant in the analysis of vitamin D_3 and its relevant metabolites.

Keywords: Serum, Plasma, Blood, Vitamin D, Hydroxymetabolites, Selected reaction monitoring.

1. Introduction

Clinical testing for vitamin D has increased exponentially in the past decade. In the United States, requests to clinical laboratories for analysis of this vitamin have increased at a rate of 80–90% per year [1]. This growing demand is a consequence of the recognition of both a high prevalence of deficiency in vitamin D in diverse social sectors [2] and the decisive role of vitamin D in multiple physiological functions. Thus, vitamin D deficiency or insufficiency has been associated to skeletal diseases such as rickets, osteomalacia and osteoporosis, but also to several non-skeletal chronic diseases including cardiovascular diseases, certain types of cancer, diabetes and psychiatric illness, among others [3].

Currently, vitamin D metabolites determined with clinical purposes are $25(OH)D_3$ (known as the circulating form of vitamin D), and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], known as the most physiologically active form of vitamin D [4]. The analysis of both metabolites can provide information on the status of vitamin D and its availability for the organism [5]. Other less studied metabolite such as 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], produced by vitamin D catabolism, is receiving growing attention from clinicians as an increase in the production of this metabolite could pinpoint an optimum balance of vitamin D [6].

The analysis of vitamin D and its metabolites is characterized by a high complexity owing to their instability in the presence of heat or UV light, their hydrophobic nature, the high affinity for vitamin D binding proteins, the structural similarity to other in circulation metabolites, and the composition of biological samples [7,8]. All these factors make the accurate measurement of vitamin D_3 a challenging task. Semiautomated and fully automated immunoassay methods have been reported; most of them based on competitive protein-binding assay (CPB) [9] — radioimmunoassay (RIA) [10,11], enzyme-linked immuno-sorbent assay (ELISA) [12], and chemiluminescence immunoassays (CLIA) [9]—, and endowed with good accuracy, mainly for $25(OH)D_3$ measurement. However, interferences caused by

cross-reactivity for monohydroxy and dihydroxymetabolites have been described [7]. The high selectivity and sensitivity of mass spectrometers allow differentiating $25(OH)D_3$ from $25(OH)D_2$, and also make possible quantitation of dihydroxy-metabolites $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, present in blood at pg/mL and ng/mL levels, respectively [6,7].

A wide variety of biological samples have been tested for analysis of vitamin D and its metabolites. A sample with low clinical interest such as saliva has been used to determine $25(OH)D_3$ and $1,25(OH)_2D_3$ metabolites [13]. Cerebrospinal fluid has also reported detectable levels of vitamin D metabolites, particularly $25(OH)D_3$ [14,15]. Dried blood spots have provided good accuracy and precision for quantitation of $25(OH)D_3$ [16,17]. Despite the studies carried out with all these samples, serum and plasma persist as the two most common for determination of vitamin D and its metabolites because both can be easily obtained and contain the most important metabolites at measurable levels. Additionally, sample preparation protocols are properly known. Protein precipitation has been mainly carried out by methanol, acetonitrile or mixtures of both solvents, followed by a separation step based on either liquid–liquid extraction (by heptane, hexane, ethyl acetate or ethyl-*tert*-butyl ether as extractants) or by solid-phase extraction (SPE) [18].

The principal aim of this research was to compare the influence of the procedure for blood collection on the determination of vitamin D and its metabolites by SPE–LC–MS/MS. With this aim, the study was focused on evaluation of two aspects that could exert a significant influence on the blood levels of vitamin D found. The first was selection of the analytical sample used for quantitative analysis: serum or plasma; the second aspect was the effect of the gel present in the blood collection tubes, which is used to favor separation of serum or plasma from blood cells on the analysis of vitamin D and its metabolites. A cohort formed by thirteen volunteers was selected for blood sampling using four different tubes (plasma, plasma-gel, serum and serum-gel). The resulting samples were analyzed by an isotopic

dilution SPE–LC–MS/MS method for absolute quantitation of vitamin D as well as its main metabolites with clinical interest. Prior to analysis, protein precipitation and SPE using an automated system were evaluated as sample preparation alternatives.

2. Experimental

2.1. Chemicals and reagents

LC–MS grade solvents were used in this research. Ammonium formate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) and acetonitrile (ACN), formic acid and methanol from Scharlab (Barcelona, Spain) were used from preparation of chromatographic mobile phases and solutions for sample preparation. Vitamins D_2 and D_3, the monohydroxymetabolites 25(OH)D_2 and 25(OH)D_3, and dihydroxy-metabolites 1,25(OH)$_2D_2$, 1,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ were from Sigma. Stable isotopic standards 1,25(OH)$_2D_3$-d_6, 24,25(OH)$_2D_3$-d_6, 25(OH)D_3-d_6, and vitamin D_3-d_6 were provided by A. Mouriño (Department of Organic Chemistry, University of Santiago, Santiago de Compostela, Spain) and M.A. Maestro (Department of Fundamental Chemistry, University of La Coruña, La Coruña, Spain), while 25(OH)D_2-d_3 and vitamin D_2-d_3 were from Sigma. Individual standard solutions were prepared by dissolving 1 mg of each analyte or isotopic standard in 10 mL of methanol, from which two solutions were prepared by dilution of the appropriate volume in methanol. One multistandard working solution was prepared with the target analytes at different concentrations: 100 ng/mL for dihydroxymetabolites 1,25(OH)$_2D_2$ and 1,25(OH)$_2D_3$; 1 mg/mL for 24,25(OH)$_2D_3$; 5 mg/mL for 25(OH)D_3 and 25(OH)D_2; and 10 mg/mL for vitamin D_2 and vitamin D_3. Other solution was prepared with each isotopic standard —7.5 mg/mL for 1,25(OH)$_2D_3$-d_6 and 125 mg/mL for 24,25(OH)$_2D_3$-d_6; 625 mg/mL for 25(OH)D_3-d_6 and 25(OH)D_2-d_3; and 625 mg/mL for vitamin D_2-d_3 and vitamin D_3-d_6. Both solutions were used for optimization, characterization and validation of the analytical methods.

2.2. Instruments and apparatus

The analyses involved reversed-phase LC (RP-LC) separation followed by electrospray ionization in positive mode (ESI+) and MS/MS detection in selected reaction monitoring (SRM). Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer. The data were processed using MassHunter Workstation Software (V-B.05) for qualitative and quantitative analysis. Hyphenated SPE was performed by a Symbiosis system (Spark Holland, Emmen, The Netherlands). This commercial equipment comprises a unit for automatic cartridge exchange (ACE), an autosampler (Reliance) furnished with a 0.2 mL sample loop and two high-pressure syringe dispensers (HPDs) for SPE solvent delivery. Peek tube of 0.25 mm i.d. (VICI, Houston, Texas, USA) was used to connect all valves of the Symbiosis unit and LCKMS/MS modules. Peek tubing of 1.0 mm i.d. and 130 cm length, and about 1 mL volume, was used to connect the Reliance to the ACE unit for mixing the serum sample and loading solution. A 10×2 mm cartridge packed with Hysphere C8 (Spark Holland) as sorbent material was used for SPE. The analytical column was a Poroshell 120 EC-C18 (2.7 mm particle size, 50×4.6 mm i.d.) from Agilent, while a guard column (2.7 mm 2 particle size, 5.0×2.1 mm i.d.), also from Agilent, was used to preserve the integrity of the analytical column.

2.3. Sampling, sample collection and storage

Thirteen individuals (3 men and 10 women) gave their informed consent for an assistance study involving quantitation of vitamin D and its main metabolites. All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki (2004), which were supervised by the ethical review board of Reina Sofia University Hospital (Córdoba, Spain) that approved the experiments. Venous blood

from each of the selected individuals was collected into four different Vacutainer® tubes (Becton Dickinson): plastic serum tubes with spray-coated silica (serum), plastic serum tubes with spray-coated silica and a polymer gel to favor serum separation (serum-gel), spray-coated silica tubes with heparin for plasma (plasma) and heparin tubes with polymer gel to favor plasma separation (plasma-gel). The gel forms a physical barrier between serum or plasma and blood cells during centrifugation, which allows setting a more efficient separation as compared to conventional tubes. All collection tubes were processed by centrifugation for 15 min at 1000×*g* for conventional tubes for serum and plasma isolation and at 2000×*g* for gel tubes. After that, the samples were placed in plastic ware tubes and stored at –80 ºC until analysis. Serum and plasma pools were prepared by mixture of aliquots from the blood donors for optimization of the methods for analysis of vitamin D and metabolites in both types of samples.

2.4. Sample preparation procedure based on precipitation of proteins

A volume of 240 mL of serum or plasma in an amber glass vial was spiked with 10 mL of the deuterated working solution —final concentration: 25 ng/mL of vitamin D_3-d_6 and vitamin D_2-d_3, 25 ng/mL of 25(OH)D_3-d_6 and 25(OH)D_2-d_3 and 5 ng/mL of 24,25$(OH)_2D_3$-d_6 and 0.3 ng/mL 1,25$(OH)_2D_3$-d_6—, immersed in an ice bath and treated for deproteination with 500 mL of 0.1% (v/v) formic acid in methanol, one of the most common solvents used for this step [19]. The vial was shaken for 5 min and the precipitate removed after centrifugation for 5 min at 4 ºC and 20,200×*g*. The upper liquid phase was collected in a vial and evaporated; then, the dry residue was reconstituted with 30 mL methanol and placed in the LC autosampler for subsequent analysis.

2.5. Sample preparation procedure based on SPE

A volume of 240 mL of serum or plasma in an amber glass vial was spiked with 10 mL of the deuterated working solution —final concentration: 25 ng/mL of vitamin

D_3-d_6 and vitamin D_2-d_3, 25 ng/mL of 25(OH)D_3-d_6 and 25(OH)D_2-d_3 and 5 ng/mL of 24,25$(OH)_2D_3$-d_6 and 0.3 ng/mL 1,25$(OH)_2D_3$-d_6—, shaken and introduced into the autosampler. Supplementary Fig. 1A shows the instrumental arrangement used for analysis of vitamin D and its metabolites. The sample loop was filled with 0.2 mL from the sample vial refrigerated at 6 ºC. The sequence of automatic operations followed in the procedure is described in Supplementary Table 1.

2.6. LC–MS/MS analysis

The LC–MS/MS method used in this study for analysis of vitamin D and metabolites was that developed by Mena-Bravo *et al*. [20]. The initial chromatographic mobile phase was 5 mM ammonium formate in 85:15 (v/v) methanol–water at a flow rate of 0.5 mL/min. The temperature of the analytical column compartment was set at 15 ºC . A linear gradient was programmed from 2 to 5 min to obtain as final composition of the mobile phase 5 mM ammonium formate in methanol, which was kept for 10 min up to the end of the chromatographic step. The total analysis time was 15 min, 10 min being required for re-establishing and equilibrating the initial conditions. The chromatographic–detection step of one sample and the SPE step of the next sample were overlapped, thus improving the analysis frequency.

The eluate from the chromatographic column was monitored by MS/MS in SRM mode. The flow and temperature of the drying gas (N_2) were 9 L/min and 350 ºC, respectively. The nebulizer pressure was 50 psi, and the capillary voltage 4750 V in positive ionization mode. The SRM parameters are specified in Supplementary Table 2 for each analyte monitored in this study as well as the isotopic standards.

2.7. Data treatment

Quantitation was carried out using the ratio between the peak area of each analyte and that of the corresponding isotopic standard. Calibration models were developed for each analyte using a pool of serum or plasma, which was spiked with different concentrations of standard solutions of the target analyte and with constant concentrations of the deuterated standards. Also, aliquots of serum or plasma spiked only with deuterated standards were prepared to correct the endogenous concentration of the target analytes in the serum or plasma pool.

3. Results and discussion

3.1. Comparison of the two sample preparation procedures

Protein precipitation and SPE were compared in terms of sensitivity, linear calibration range and matrix effects for analysis of vitamin D and its metabolites. For this purpose, four calibration models were built for each analyte using serum and plasma and the two sample preparation methods. The calibration models were prepared using pools of both biofluids from donors, which were spiked with the target analytes at different concentrations. Table 1 lists the main parameters of the resulting calibration models, the calibration ranges of which were defined according to the normal blood levels of each analyte described in the literature [18].

The upper value of the linear dynamic range was the same for both sample preparation alternatives, 500 ng/mL for vitamin D_2 and D_3 and 250 ng/mL for the two monohydroxymetabolites. Nevertheless, the lower limits of the linear dynamic ranges were clearly influenced by the sample preparation approach.

Table 1. Features of the calibration models for analysis of vitamin D and metabolites in serum and plasma as a function of sample preparation.

Vit-D_3	Sample	Calibration range	R^2	Equation
SPE	Serum	500 ng/mL –1 pg/mL	98.75%	y = 1.276x –1.5581
	Plasma	500 ng/mL –1 pg/mL	99.72%	y = 1.3693x –0.8741
P.P.	Serum	500 ng/mL –1.5 ng/mL	99.52%	y = 0.1122x – 0.1758
	Plasma	500 ng/mL –1.5 ng/mL	98.82%	y = 0.1096x – 0.0339
Vit-D_2	Sample	Calibration range	R^2	Equation
SPE	Serum	500 ng/mL –50 pg/mL	99.23%	y = 0.1716x – 0.4733
	Plasma	500 ng/mL –50 pg/mL	99.90%	y = 0.1662x + 0.2503
P.P.	Serum	500 ng/mL –1.5 ng/mL	98.96%	y = 0.0731x – 0.2493
	Plasma	500 ng/mL –1.5 ng/mL	96.31%	y = 0.06x + 0.0738
25(OH)D_3	Sample	Calibration range	R^2	Equation
SPE	Serum	250 ng/mL –1 pg/mL	98.08%	y = 0.5148x – 0.4278
	Plasma	250 ng/mL –1 pg/mL	99.87%	y = 0.1542x + 0.4620
P.P.	Serum	250 ng/mL –0.75 ng/mL	98.63%	y = 0.0285x + 0.032
	Plasma	250 ng/mL –0.75 ng/mL	98.34%	y = 0.0331x + 0.0483
25(OH)D_2	Sample	Calibration range	R^2	Equation
SPE	Serum	250 ng/mL –0.25ng/mL	98.66%	y = 0.6219x – 0.5481
	Plasma	250 ng/mL –0.25ng/mL	99.72%	y = 0.3908x + 0.7616
P.P.	Serum	250 ng/mL –0.75ng/mL	98.96%	y = 0.019x + 0.1157
	Plasma	250 ng/mL –0.75ng/mL	98.97%	y = 0.0261x – 0.0293
24,25$(OH)_2D_3$	Sample	Calibration range	R^2	Equation
SPE	Serum	50 ng/mL –15pg/mL	99.59%	y = 1.7943x – 0.564
	Plasma	50 ng/mL –15pg/mL	99.38%	y = 3.913x – 0.8856
P.P.	Serum	50 ng/mL –0.15ng/mL	96.96%	y = 0.1013x + 0.0852
	Plasma	50 ng/mL –0.15ng/mL	96.84%	y = 0.1918x + 0.1188
1,25$(OH)_2D_2$	Sample	Calibration range	R^2	Equation
SPE	Serum	50 ng/mL –50pg/mL	99.10%	y = 2.0654x – 0.4258
	Plasma	50 ng/mL –50pg/mL	99.73%	y = 2.8137x + 1.7614
P.P.	Serum	50 ng/mL –0.15ng/mL	98.31%	y = 0.1603x + 0.2784
	Plasma	50 ng/mL –0.15ng/mL	96.47%	y = 0.2676x + 0.1188
1,25$(OH)_2D_3$	Sample	Calibration range	R^2	Equation
SPE	Serum	5 ng/mL –15pg/mL	99.16%	y = 0.0013x + 0.1445
	Plasma	5 ng/mL –15pg/mL	98.89%	y = 0.0043x – 0.09
P.P.	Serum	5 ng/mL –0.1ng/mL	96.49%	y = 0.1835x + 0.0116
	Plasma	5 ng/mL –0.1ng/mL	94.04%	y = 0.0002x + 0.1976

Thus, protein precipitation both in plasma and serum led to higher limits of quantitation than those provided by SPE. The most compromised situation was found for the three dihydroxymetabolites —$1,25(OH)_2D_2$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$—, since the protein precipitation method provided limits of quantitation (LOQs) from 0.1 to 0.15 ng/mL, considerably above the typical levels of these metabolites in blood. On the other hand, the LOQs were 15 pg/mL for the two dihydroxymetabolites of vitamin D_3, while for $1,25(OH)_2D_2$ the LOQ was 50 pg/mL. As can be checked, vitamin D_2 and its metabolites gave LOQs higher than their vitamin D_3 analogues.

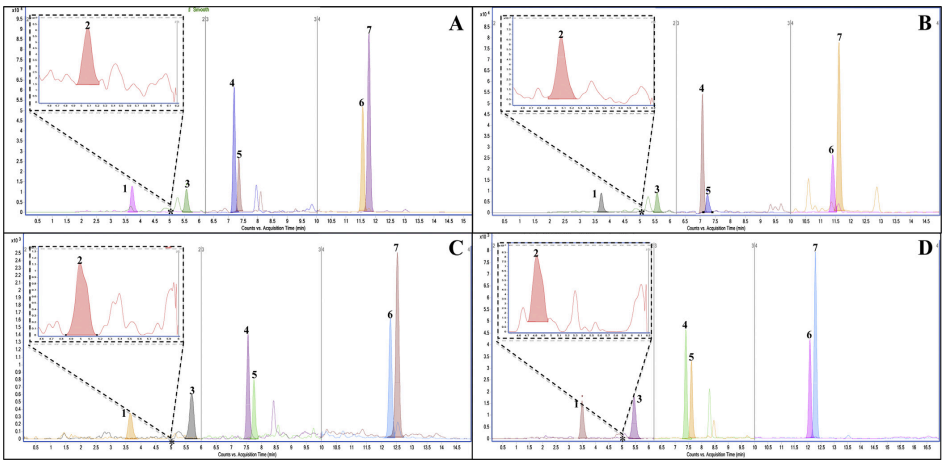

Fig. 1. (A) SRM serum with SPE method, (B) SRM plasma with SPE method, (C) serum with de PP method and (D) Plasma with PP method. (1) $24,25(OH)_2D_3$, (2) $1,25(OH)_2D_3$, (3) $1,25(OH)_2D_2$, (4) $25(OH)D_3$, (5) $25(OH)D_2$, (6) vitamin D_2 and (7) vitamin D_3.

This different sensitivity should be attributed to the better electrospray ionization efficiency for vitamin D_3 and its metabolites that could be explained by the higher hydrophobic character of vitamin D_2 and its metabolites. Figure 1 shows the SRM chromatograms obtained by analysis of the pools of serum and plasma spiked with

the target analytes at intermediate concentrations [18] by protein precipitation and SPE–LC–MS/MS.

According to the obtained LOQs and linear calibration ranges, deproteination can only be implemented in methods targeted at the analysis of vitamin D and its metabolites, except $1,25(OH)_2D_3$. The differences in sensitivity can be justified by the volume of sample injected on-column in each approach and, thus, by the preconcentration effect: 41.6 mL in protein precipitation (by considering all the steps involved in this sample preparation procedure) versus 200 mL for the SPE-based method, 4.8 times higher in SPE.

3.2. Differences between serum and plasma for SPE–LC–MS/MS analysis of vitamin D

Once the best performance of the SPE method for analysis of vitamin D was assured, the influence of the type of sample, plasma or serum, on the optimum values of the variables that affect the analysis of vitamin D and metabolites was assessed. The chemical composition of serum and plasma allowed establishing differences in the loading of the sample and the wash of the SPE cartridge. Thus, the composition of the loading solution for serum and plasma was the same: 25% (v/v) ACN in water acidified with formic acid, but the concentration of the acid was 0.7% (v/v) for serum and 0.5% (v/v) for plasma.

Concerning the cartridge washing, a slight difference was also observed in the used solution, 30% (v/v) ACN in water for serum and 20% (v/v) for plasma. Differences in the loading and washing steps of the SPE process should be strongly associated to the matrix composition of both samples. The major difference between plasma and serum is the removal of fibrinogen and associated proteins by the coagulation process. The absence of these proteins allows increase the concentration of organic solvent and formic acid in the SPE process for analysis of serum, while

these concentrations enhance protein precipitation in plasma. Concerning the calibration models built for serum and plasma, particular differences were found in the slopes of the calibration equations for some metabolites, as Table 1 shows. These differences allow establishing a comparison in terms of sensitivity of the method. Thus, the sensitivity was significantly better for the dihydroxymetabolites in plasma as compared to serum; while the opposite effect was observed for the two monohydroxymetabolites. The different sensitivity associated to the type of sample could be attributed to matrix interferents that could exert influence on each at a particular retention time.

The SPE–LC–MS/MS methods for analysis of vitamin D and metabolites in serum and plasma were compared in terms of recovery, accuracy and precision. These analytical features were calculated by using pools of serum and plasma spiked at three concentrations (see Table 2) representing low, intermediate and high concentrations of the target analytes, according to the literature [18].

Table 2. Comparison of recovery in serum and plasma by the SPE method.

	Recovery (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	60.1	59.0	98.8	97.2	100	100	99.8
Plasma	58.6	56.4	97.2	83.0	100	100	99.7

The configuration of the SPE approach used for the recovery estimation was based on the coupling of two cartridges, as Supplementary Fig. 1.B illustrates [21–23]. The average values obtained for both types of samples at the three spiked concentrations are shown in Table 3.

Table 3. Differences between serum and plasma in accuracy analyses by the SPE method.

	Accuracy (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	93.5	83.3	96.3	89.3	89.3	95.9	90.9
Plasma	90.1	104.1	96.4	95.6	96.9	97.5	105.1

The recovery factor was practically 100% for the three monitored dihydroxy-metabolites, either in serum or plasma. A slight decrease in the recovery factor was observed for the monohydroxymetabolites as their retention time increased, which was more significant in the case of 25(OH)D_2 when determined in plasma. Thus, the recovery factor was 98.8 and 97.2% for 25(OH)D_3 in serum and plasma, respectively; while this parameter was 97.2 and 83.0% for 25(OH)D_2 in serum and plasma, respectively. The decrease of the recovery factor as the retention time increased was more significant for vitamins D_2 and D_3, as this parameter was around 60% both in serum and plasma. The higher polarity of the didydroxymetabolites as compared to the monohydroxymetabolites and the two forms of vitamin D allows obtaining an efficient retention of the dihydroxylated forms, which is of paramount importance to address their quantitative analysis taking into account their low levels in biological samples.

The accuracy and potential matrix effects were studied by the configuration described in Supplementary Fig. 1.A. The average accuracy values were above 89% for all analytes in the case of serum, except for vitamin D_2 that yielded 83.3% (see Table 3). For plasma, the accuracy values were above 90.1%. In general, the accuracy calculated for most analytes was slightly better in plasma than in serum, except for vitamin D_3.

The precision for each type of sample was evaluated under two experimental conditions: within-day variability and between-day variability. For this purpose, a single experimental set-up with duplicate analysis per day was carried out with pools of serum and plasma spiked with the target analytes at intermediate levels (see Table 2) for 7 days. The results thus obtained are summarized in Table 4, showing that the precision, expressed as relative standard desviation, was below 12.9%, and this analytical feature was not influenced by the type of sample: serum or plasma.

Table 4. Differences between serum and plasma in "within and between days" analyses with SPE method.

	Within day (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	2.1	6.0	7.2	4.4	3.2	3.9	6.4
Plasma	3.9	11.5	4.5	2.0	1.5	2.9	8.0
	Between days (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	11.6	8.9	9.9	5.3	8.2	7.8	11.3
Plasma	7.1	12.9	8.3	3.5	2.6	9.1	8.9

3.3. Influence of the tube used for blood collection

The influence of the blood collection tube on the determination of vitamin D and metabolites was evaluated by analysis of serum and plasma samples collected in conventional tubes (spray-coated silica tubes) and in polymer-gel tubes. The concentrations of the target analytes found in the four types of samples obtained from the target cohort are listed in Table 5.

Table 5. Minimum, maximum and mean concentrations of the target analytes found in four types of samples (collected in serum, serum-gel, plasma and plasma-gel tubes) from the selected cohort.

Serum	Minimum	Maximum	Mean
$1,25(OH)_2D_3$ (pg/mL)	50.3	111.6	103.7
$24,25(OH)_2D_3$ (ng/mL)	2.0	7.9	4.3
$25(OH)D_3$ (ng/mL)	13.8	45.9	29.2
Vitamin D_3 (ng/mL)	4.9	11.4	7.3
Serum-gel	Minimum	Maximum	Mean
$1,25(OH)_2D_3$ (pg/mL)	51.7	174.4	109.2
$24,25(OH)_2D_3$ (ng/mL)	1.3	6.7	4.2
$25(OH)D_3$ (ng/mL)	20.6	46.4	30.0
Vitamin D_3 (ng/mL)	5.0	9.5	7.3
Plasma	Minimum	Maximum	Mean
$1,25(OH)_2D_3$ (pg/mL)	160.5	228.1	160.3
$24,25(OH)_2D_3$ (ng/mL)	1.5	6.4	4.3
$25(OH)D_3$ (ng/mL)	15.9	49.2	31.5
Vitamin D_3 (ng/mL)	4.7	10.3	6.9
Plasma-gel	Minimum	Maximum	Mean
$1,25(OH)_2D_3$ (pg/mL)	73.4	235.0	152.8
$24,25(OH)_2D_3$ (ng/mL)	1.9	7.9	4.3
$25(OH)D_3$ (ng/mL)	15.4	41.3	31.5
Vitamin D_3 (ng/mL)	4.3	9.4	6.4

A paired *t*-test (95% confidence level) was applied to check the existence or absence of statistical differences between levels of the target analytes in serum or plasma collected in conventional and gel tubes. It is worth mentioning that this study was based on the determination of vitamin D_3 and metabolites —25OHD3, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$— since vitamin D_2 and metabolites were not detected in the volunteers of the cohort as they were not receiving vitamin D supplement. In the case of serum, conventional and gel tubes could be indistinctly used for quantitative analysis of vitamin D. On the contrary, plasma analysis led to

detect statistical differences in the analysis of vitamin D_3 depending on collection, which is currently out of the scope of clinical tests. Figure 2 shows that the levels of vitamin D_3 in plasma tubes were always higher than in plasma-gel tubes. Therefore, the use of plasma and plasma-gel tubes is not critical for quantitative determination of vitamin D metabolites, which are the main objective from a clinical perspective.

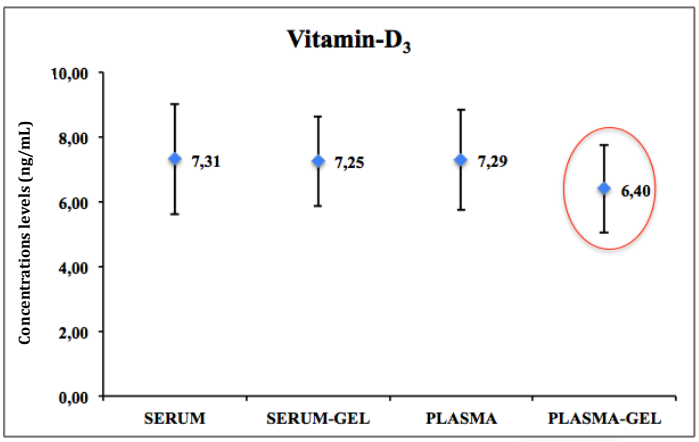

Fig. 2. Concentration of vitamin D_3, expressed as ng/mL, in samples from the selected cohort collected by using the four types of tubes.

3.4. Comparison of the use of plasma or serum for quantitative analysis of vitamin D

The levels of vitamin D_3 and metabolites in plasma and serum samples of the cohort were also statistically compared by the paired *t*-test (95% confidence level). Figure 3 shows the concentration ranges of vitamin D_3 and metabolites — particularly, $25OHD_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$— found in the cohort of

volunteers. The *t*-test allowed detecting no statistical differences between serum and plasma levels of vitamin D_3, $25(OH)D_3$ and $24,25(OH)_2D_3$ metabolites, but statistical differences between levels of $1,25(OH)_2D_3$ measured in serum and plasma.

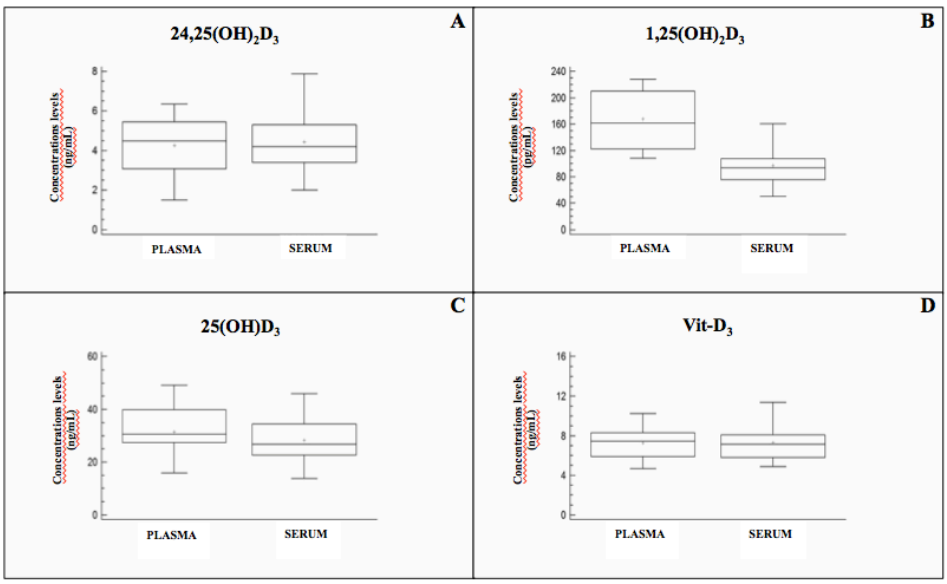

Fig. 3. Box-and-whisker plots presenting the concentrations of (A) $24,25(OH)_2D_3$, (B) $1,25(OH)_2D_3$, (C),$25(OH)D_3$ and (D) vitamin D_3 found in plasma and serum from the cohort of volunteers.

Thus, the concentration of this metabolite, which is characterized by lower concentrations than the rest of the metabolites, varied from 111.5 to 226.2 pg/mL in plasma; while its range was quite lower in serum: from 52.7 to 152.6 pg/mL. Therefore, plasma seems to be more suited than serum for quantitative analysis of $1,25(OH)_2D_3$, which is important taking into account that quantification of this dihydroxymetabolite is the most limiting aspect of methods for assessment of vitamin D status.

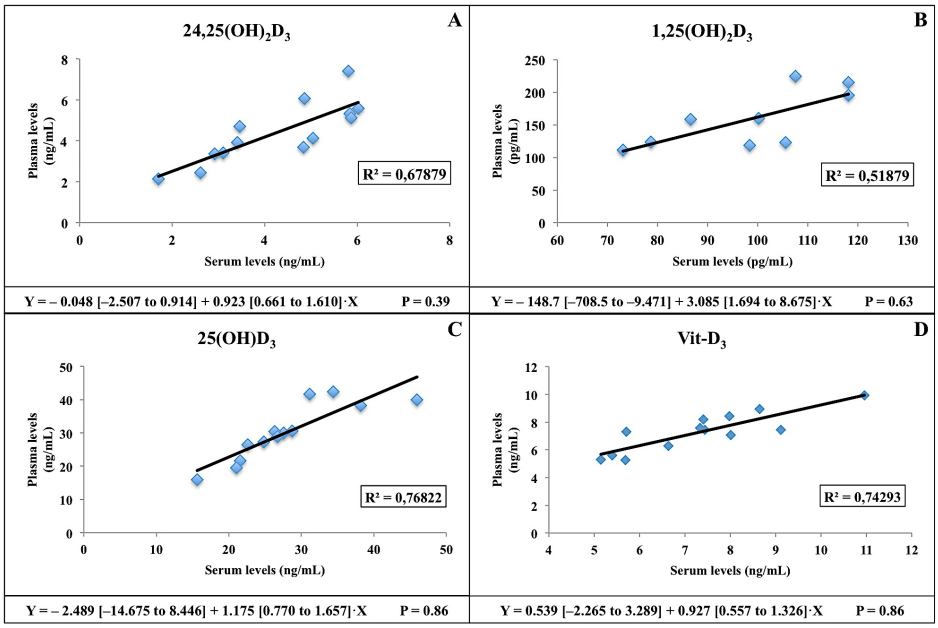

Fig. 4. Passing–Bablok regression analysis for levels of (A) 24,25(OH)$_2$D$_3$, (B) 1,25(OH)$_2$D$_3$, (C) 25OHD3 and (D) vitamin D$_3$ obtained in serum and plasma from the volunteers.

A Passing–Bablok regression analysis [24] for each analyte was carried out with the levels measured in serum and plasma of the volunteers. Figure 4 illustrates the regression graphs thus obtained, as well as the values of the main statistical parameters. The regression coefficients ranged from 0.518 for 1,25(OH)$_2$D$_3$ to 0.768 for 25(OH)D$_3$. The validity of the linear models was studied by the Cusum test, which reported no significant deviation from linearity for vitamin D$_3$ and its three metabolites (*p*-value of 0.39 for 24,25(OH)$_2$D$_3$ and above 0.86 for the rest of the analytes).

4. Conclusions

The developed research has allowed elucidation of key aspects on collection and preparation of blood for analysis of vitamin D and its metabolites. Thus, sample preparation based on SPE provides lower quantitation limits for all the target analytes than deproteination.

The type of sample influences the sensitivity of the method since plasma is better for determination of the dihydroxymetabolites, while the two monohydroxymetabolites are determined with better sensitivity in serum.

Statistical comparison of the use of plasma or serum as target sample has shown that the concentration of $1,25(OH)_2D_3$ in plasma was higher than that in serum. This finding is important taking into account that quantitation of this dihydroxymetabolite is the most limiting aspect of methods for assessment of vitamin D status. The use of collection tubes with or without coating gel shows significant statistical differences only for vitamin D_3 in plasma, which yielded a higher concentration when the sample was collected in conventional plasma tubes as compared with plasma-gel tubes.

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Supplementary Table 1.A. Steps programmed in the serum SPE protocol.

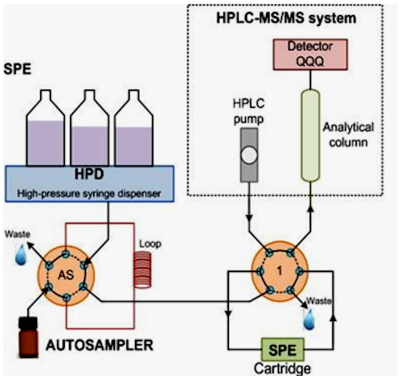
Step	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				
Start autosampler				Load sample
Solvation 1	10	2	Methanol	
Solvation 2	5	4	Methanol	
Equilibration	3	4	25% ACN–0.7%F	
Equilibration	3	4	25% ACN–0.7%F	
Sample application	3	4	25% ACN–0.7%F	
Wash cartridge	3	0.5	30% ACN	
Elution			Mobile phase	5 min
Purge 1	5	5	Methanol	
Purge 2	5	5	30% ACN	
Purge 3	5	5	Water	
Purge 4	5	5	30% ACN	
Purge 5	5	5	Water	

Supplementary Table 1.B. Steps programmed in the plasma SPE protocol.

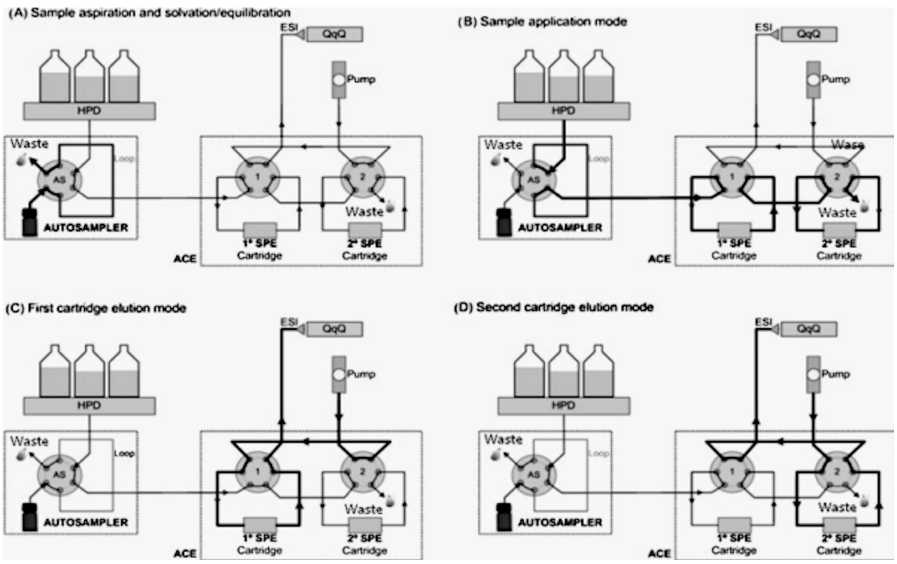
Step	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				
Start autosampler				Load sample
Solvation 1	10	2	Methanol	
Solvation 2	5	4	Methanol	
Equilibration	3	4	25% ACN–0.5%F	
Equilibration	3	4	25% ACN–0.5%F	
Sample application	3	4	25% ACN–0.5%F	
Wash cartridge	3	0.5	20% ACN	
Elution			Mobile phase	6 min
Purge 1	5	5	Methanol	
Purge 2	5	5	20% ACN	
Purge 3	5	5	Water	
Purge 4	5	5	20% ACN	
Purge 5	5	5	Water	

Supplementary Table 2. Concentrations of the target analytes spiked in serum and plasma for estimation of the recovery factor and for the accuracy study.

Analyte	Low level (ng/mL)	Intermediate level (ng/mL)	High level (ng/mL)
$1,25(OH)_2D_3$	0.05	0.1	0.2
$1,25(OH)_2D_2$	3	10	20
$24,25(OH)_2D_3$			
$25(OH)D_3$	15	50	100
$25(OH)D_2$			
Vit D_2	15	50	100
Vit D_3			



Supplementary Fig. 1. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method.



Supplementary Fig. 2. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method for estimation of the recovery factor. (A) Sample aspiration and solvation/equilibration. (B) Sample application mode. (C) First cartridge elution mode. (D) Second cartridge elution mode.

Capítulo 4

Evaluation of human serum storage prior to analysis of vitamin D3 and metabolites by liquid chromatography coupled to tandem mass spectrometry

(sent for publication)

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(sent for publication)

Evaluation of human serum storage prior to analysis of vitamin D_3 and metabolites by liquid chromatography coupled to tandem mass spectrometry

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Abstract

Vitamin D has been widely determined in clinical trials to elucidate its biochemical involvement in a great number of pathologies. The analysis of vitamin D and its hydroxymetabolites in biofluids such as serum or plasma is a challenging task due to limitations associated to the low concentrations of some metabolites (typically, dihydroxymetabolites), methodological interferences, and the low stability of the compounds. Among these limitations, efforts have been targeted at optimizing instrumental improvements to develop more sensitive and selective methods, while the stability of vitamin D and metabolites has not been exhaustively evaluated. In this research, several aspects regarding stability of vitamin D_3 and metabolites in serum have been studied by testing different sample storage conditions. An experimental plan has been applied to assess the influence on vitamin D_3 stability of two relevant parameters: the storage temperature for a period of two months and the number of freeze/thaw cycles. The storage

temperature affected in a different manner to vitamin D_3 and its metabolites, being vitamin D_3 and 1,25-dihydroxyvitamin D_3 the two analytes more affected by this parameter. Concerning the freeze/thaw cycles, this variable must be limited to two cycles owing to its significant influence on the stability in serum of the two dihydroxymetabolites. Finally, lyophilization was also tested to check if serum concentrations of vitamin D_3 and its metabolites were affected by this preprocessing step. The results revealed that only vitamin D_3 decreased its concentration in serum after two months, which does not constitute a real problem, as vitamin D_3 is not currently a crucial parameter to be determined in clinical trials due to its scant biological activity.

Keywords: Vitamin D; 25-Monohydroxyvitamin D; 1,25-Dihydroxyvitamin D; 24,25-Dihydroxyvitamin D; Stability; Liquid chromatography; Mass spectrometry.

1. Introduction

Vitamin D is a steroid prohormone found in the human body in two main forms: vitamin D_3 (cholecalciferol) is synthesized from 7-dehydrocholesterol as precursor in human skin through a process initiated by sun exposure; vitamin D_2 (ergocalciferol) is obtained from the diet or as a supplement [1]. Vitamin D is involved in a wide number of biological functions and its receptors are found in different tissues (bones, intestine, kidney, etc.) [2]. Focused on vitamin D_3, this vitamin is converted in the liver by a 25-hydroxylation process to form 25-hydroxyvitamin D_3 [$25(OH)D_3$], the major circulating form and principal regulator of calcium and phosphorus homeostasis [3]. $25(OH)D_3$ is metabolized in the kidney and extra renal sites into 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], known as the active form, and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], which is identified as one of the first metabolites found in the excretion of vitamin D [1–3].

More than 1600 studies have been developed during the past decade about vitamin D and its connection to health. More than a half showed that vitamin D deficiency is associated with a wide variety of chronic disorders [4], such as diabetes, multiple sclerosis [5], cardiovascular diseases, asthma [3], cancer (lung, colorectal, breast or prostate cancer [6]), Parkinson [7] and cystic fibrosis [8]. The concentration of the circulating form, $25(OH)D_3$, was the target biomarker in these epidemiological studies [6]. Nevertheless, strong anti-proliferative and cell differentiation-inducing activities have been recognized for $1,25(OH)_2D_3$, apart from its role in calcium homeostasis [9]. Among vitamin D metabolites, $1,25(OH)_2D_3$ has been preferentially used for in vitro experiments that have provided several evidences of potential action mechanisms for vitamin D in carcinogenesis [6]. On the other hand, the $25(OH)D_3/24,25(OH)_2D_3$ ratio has recently been proposed as a potential indicator of vitamin D status in human body [10].

Recent studies on dihydroxyvitamin D metabolites, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, have shown the need for a complete profile of these compounds to estimate the vitamin D status in each individual. For this purpose, different quantitative methods for analysis of vitamin D metabolites have been published, with special predominance of those based on immunoassay and LC–MS/MS [11,12,13]. Nevertheless, LC–MS/MS is considered the gold standard due to its high sensitivity (ng/mL and pg/mL) and selectivity, which can be clearly improved by the use of chemically labeled isotopic standards. On the other hand, immunoassay methods suffer frequently from cross-reactivity effects that alter absolute quantification.

The stability of these analytes in clinical samples, with special emphasis on serum/plasma, is a key aspect for a reliable assessment of the quantitative results in epidemiological studies. The behavior of the target analytes from blood extraction to determination of vitamin D and metabolites has a huge relevance, considering their low concentration in the human body (ng/mL or pg/mL levels). Additionally, there are factors such as the periodical season that affects significantly to the concentration of vitamin D and metabolites. The stability of vitamin D metabolites in serum has been insufficiently studied so far. In fact, only the stability of $25(OH)D_3$ has been matter of study [2,14–18], while the rest of metabolites have been ignored. In addition, the stability studies dealing with $25(OH)D_3$ were programmed for short time periods [2] or the method for evaluation was not the "*gold standard"* based on mass spectrometry [15,17].

The principal aims of this research were to study the stability of vitamin D_3 and its main metabolites —$25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$— in human serum. Five strategies for sample storage (room temperature, refrigerated at 4 ºC, frozen at –20 ºC or at –80 ºC, and lyophilization) were tested for two months. The influence of freeze/thaw cycles on the concentration of the target analytes was also evaluated.

2. Experimental

2.1. Chemicals and reagents

LC–MS grade solvents were used in this research. Ammonium formate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) and acetonitrile (ACN), formic acid and methanol from Scharlab (Barcelona, Spain) were used for preparation of chromatographic mobile phases and for sample preparation.

Vitamin D_3, the monohydroxymetabolite $25(OH)D_3$, and dihydroxymetabolites $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were from Sigma–Aldrich. Stable isotopic standard $24,25(OH)_2D_3$-d_6 was provided by A. Mouriño (Department of Organic Chemistry, Faculty of Chemistry, University of Santiago, Santiago de Compostela, Spain) and M. A. Maestro (Department of Fundamental Chemistry. Faculty of Sciences, University of La Coruña, La Coruña, Spain), while $1,25(OH)_2D_3$-d_6, $25(OH)_2D_3$-d_3, and vitamin D_3-d_3 were from Sigma–Aldrich. Individual standard solutions were prepared by dissolving 1 mg of each analyte or isotopic standard in 10 mL of methanol. One multistandard working solution was prepared with the target analytes at different concentrations: 100 ng/mL for $1,25(OH)_2D_3$, 1 μg/mL for $24,25(OH)_2D_3$, 5 μg/mL for $25(OH)D_3$, and 10 μg/mL for vitamin D_3. These concentrations were selected according the typical physiological levels found in humans [1]. One other solution was prepared with the isotopic standards — $1,25(OH)_2D_3$-d_6 at 7.5 ng/mL, $24,25(OH)_2D_3$-d_6 at 125 μg/mL, $25(OH)_2D_3$-d_3 at 625 μg/mL, and vitamin D_3-d_3 at 625 ng/mL. Both solutions were used to spike serum aliquots from a pool at different concentration levels, which were analyzed to build calibration curves.

2.2. Instruments and apparatus

The analyses involved reversed-phase LC (RP-LC) separation followed by electrospray ionization in positive ionization mode (ESI+) and MS/MS detection by

multiple reaction monitoring (MRM). Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system coupled to an Agilent 6410 QQQ mass spectrometer. The data were processed using MassHunter Workstation Software (V-B.05) for qualitative and quantitative analysis. Hyphenated SPE was performed by a Symbiosis system (Spark Holland, Emmen, The Netherlands). This commercial equipment comprises a unit for automatic cartridge exchange (ACE), two high-pressure syringe dispensers (HPDs) for SPE solvent delivery, and an autosampler furnished with a 0.2 mL sample loop. Peek tube of 0.25 mm i.d. (VICI, Houston, Texas, USA) was used to connect all valves of the Symbiosis unit and LC–MS/MS modules. Peek tubing of 1.0 mm i.d. and 130 cm length, about 1 mL volume, was used to connect the autosampler to the ACE unit for mixing the serum sample and loading solution. For SPE, 10×2 mm cartridges packed with Hysphere C8 (Spark Holland) as sorbent material were used. The analytical column was a Poroshell 120 EC-C18 (2.7 μm particle size, 50×4.6 mm i.d.) from Agilent, and a guard column (2.7 μm particle size, 5.0×2.1 mm i.d.), also from Agilent, was used to preserve the integrity of the analytical column.

2.3. Sampling and sample collection

Blood samples were extracted from volunteers (n = 5) who gave informed consent for this assistance study involving quantitation of vitamin D and its main metabolites. Venous blood was collected into plastic serum Vacutainer® tubes (Becton Dickinson) with spray-coated silica and a polymer gel to favor serum separation. All collection tubes were immediately processed by centrifugation for 15 min at 2000×g for gel tubes. A serum pool was prepared by mixing aliquots from blood donors.

All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki (2004), which were supervised by the ethical review board of Reina Sofia University Hospital (Córdoba, Spain) that approved the experiments.

2.4. Sample storage

Aliquots of spiked samples were placed in plastic ware tubes and stored under four different conditions until analysis: (1) at room temperature, 25 ºC, preserved from sunlight; (2) in a refrigerator, 6 ºC; and in freezers, (3) at –20 ºC and, (4) at –80 ºC. Three aliquots stored under different conditions were defrosted according to a planned programme at days 0, 1, 2, 3, 5, 8, 10, 21, 30 and 60 after the beginning of the study.

Additionally, twelve aliquots were used for the study dealing with the influence of freeze/thaw cycles study. Six aliquots were stored at –20 ºC and the rest six at –80 ºC. Finally, six aliquots were frozen at –80 ºC and lyophilized with a vacuum freeze dryer (Telstar-LyoQuest, Telstar, Terrasa, Spain) to constant weight, then stored for 1 and 2 months at room temperature.

2.5. Protocol for analysis of vitamin D_3 and metabolites

The method for analysis of vitamin D_3 and its main metabolites —$25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)2D_3$— was that proposed in previous publications [19,20]. The protocol is based on an SPE–LC–MS/MS approach with direct analysis of serum without previous protein precipitation. This protocol is detailed as Supplementary Material. The instrumental arrangement used for analysis of vitamin D is included as Supplementary Fig. 1, while the sequence of automatic operations followed in the protocol is listed in Supplementary Table 1.

Mass spectrometry detection was performed by MRM using quantitative transitions for each analyte. Parameters of the MRM method are included in Table 1.

Table 1. Optimization of the MS/MS MRM method for quantitative and confirmatory analysis of vitamin D_3 and its metabolites, and internal standards.

Analyte	Precursor ion (*m/z*)	Adducts	Product ion (*m/z*)	Collision energy (eV)	Internal standard	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
$24,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	121.1	30	$24,25(OH)_2D_3$-d_6	405.4	121.1	20
$1,25(OH)_2D_3$	399.3	$[M+H-(H_2O)]^+$	227.1	20	$1,25(OH)_2D_3$-d_6	405.4	227.1	20
$25(OH)D_3$	383.3	$[M+H-(H_2O)]^+$	107.1	30	$25(OH)D_3$-d_6	389.3	107.1	25
Vit D_3	385.3	$[M+H]^+$	107.1	30	Vit D_3-d_3	385.3	107.1	25

2.6. Data treatment

Quantitation was carried out using the ratio between the peak area of each analyte and that of the corresponding isotopic standard. Calibration models were developed for each analyte using aliquots of the serum pool spiked at different concentrations with the multistandard solution of the target analytes and with constant concentrations of the deuterated standards. A blank serum spiked only with deuterated standards was prepared to correct the endogenous concentration of the target analytes in the serum pool.

For statistical analysis, the *stats* package included in R statistical language (version 3.4.3, http://www.r-project.org/) was used for *t*-test analysis by using the interface RStudio (version 1.1.423, http://www.rstudio.com).

3. Results and discussion

3.1. Influence of storage conditions on the concentration of vitamin D metabolites

The stability study was carried out by taking as reference the concentrations of the analytes determined in the serum aliquots (n=5) immediately after preparation (day 0). The concentration of each analyte in the serum pool with standard deviation is listed in Table 2. For statistical analysis, the between-days variability was selected as cut-off value with a 95% confidence level (*p*-value=0.05). This variability was in all cases lower than the 25%, error limit imposed by external evaluation programs such as the Vitamin D External Quality Assurance Scheme (DEQAS), which is targeted at vitamin D metabolites, $25(OH)D_3$ and $1,25(OH)_2D_3$. If this limit is surpassed for any analyte, vitamin D_3 or metabolites, this fact means that there is an alteration in the concentration level owing to lack of stability.

Table 2. Concentrations of the target analytes found at day 0**.**

Analyte	Day 0	SD
$1,25(OH)_2D_3$ (pg mL^{-1})	282.5	±2.02
$24,25(OH)_2D_3$ (ng mL^{-1})	10.1	±0.25
$25(OH)D_3$ (ng mL^{-1})	35.7	±1
Vitamin D_3 (ng mL^{-1})	21	±0.8

The storage time played a relevant role in the determination of vitamin D_3 and significant differences were observed after 5 days at room temperature and after 10 days under cooling in the refrigerator or freezer. The circulating $25(OH)D_3$ form did not experience significant concentration changes ($p<0.05$) under the tested temperature conditions during the preset time, as Figure 1.A shows.

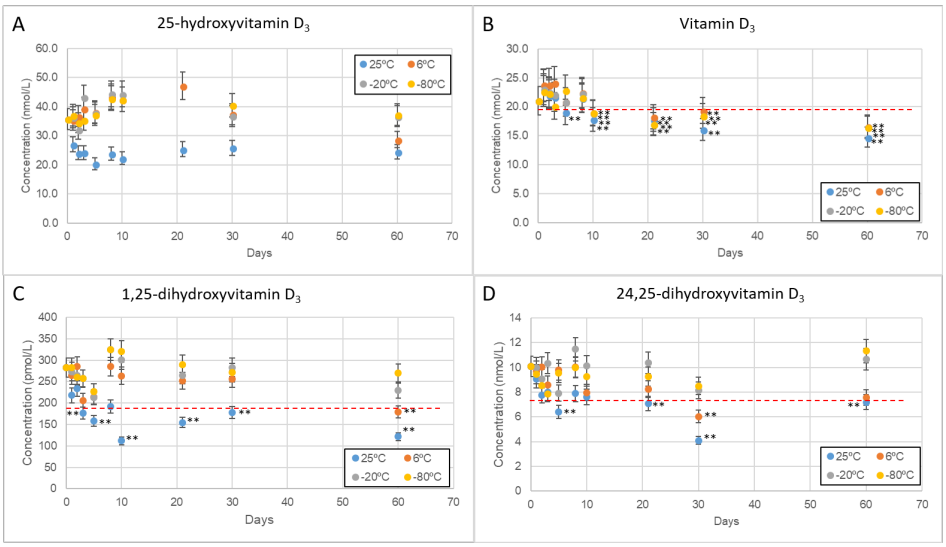

Fig. 1. Influence of storage temperature on serum concentrations of 25-hydroxyvitamin D_3 (A), vitamin D_3 (B), 1,25-dihydroxyvitamin D_3 (C) and 24,25-dihydroxyvitamin D_3 (D) versus storage time.

A slight increasing in concentration was observed when the samples were refrigerated or frozen during the first days since sampling, but this variation did not result significant at statistical level. Therefore, the circulating form was stable for two months independently of the storage temperature. This fact ensures the stability of 25(OH)D_3, which is the main determined metabolite to assess the status of vitamin D_3 in clinical trials. As Figure 1.B shows, the concentration of vitamin D_3 experienced in general a significant decrease when the storage time increased. This significant decrease was firstly detected at room temperature, but the refrigerator/freezer did not avoid the decrease of the concentration. However, this is not a limitation for interpretation of vitamin D_3 metabolic status since vitamin D_3 is usually not included in clinical studies dealing with the role of vitamin D metabolism.

The active form $1,25(OH)_2D_3$ reported the behavior illustrated in Figure 1.C, which reveals that it is possible to store serum in a freezer for two months without significant ($p<0.05$) concentration losses. A similar behavior was observed for serum stored in the refrigerator, but the aliquot analyzed after two months led to significant concentrations decreases as compared to the initial level. On the other hand, the experiment based on room temperature storage led to concentration variations at 3rd day ($p<0.05$) as compared to levels found at day 0. The low serum levels at which this metabolite is detected in human serum (pg/mL) justify the control of sample storage to avoid variations in the reported concentrations. In fact, this instability could be one of the reasons why this metabolite is frequently undetected in serum or plasma.

The determination of $24,25(OH)_2$ vitamin D_3 is gaining clinical interest due to its metabolic implications [21]. The quantitative analysis of $24,25(OH)_2$ vitamin D_3 was not affected in this statistical context when the sample was frozen at –20 ºC or –80 ºC. However, a decay of concentration was observed in aliquots kept at room temperature since the fifth day. Concerning the use of a refrigerator, a decreasing trend in the concentration level of this dihydroxymetabolite was observed, with statistical variations at 30th and 60th days since the beginning of the trial. This situation is illustrated in Figure 1.D.

3.2. Influence of freeze-thaw cycles on the quantitative analysis of vitamin D_3 and its metabolites

A complementary study involving five freeze/thaw cycles of serum aliquots of the pool stored in the freezer at two different temperatures (–20 ºC and –80 ºC) was carried out. Figure 2 shows the alterations experienced by each metabolite, taking also as reference the levels found at day 0. As can be seen, vitamin D_3 and $25(OH)D_3$ did not experience significant quantitative alterations after five freeze/thaw cycles at any of the two storage temperatures. The relative high

concentrations of 25(OH)D_3 found in human serum (at the nmol/mL) seem to be the main factor contributing to explain the reduced changes in concentration.

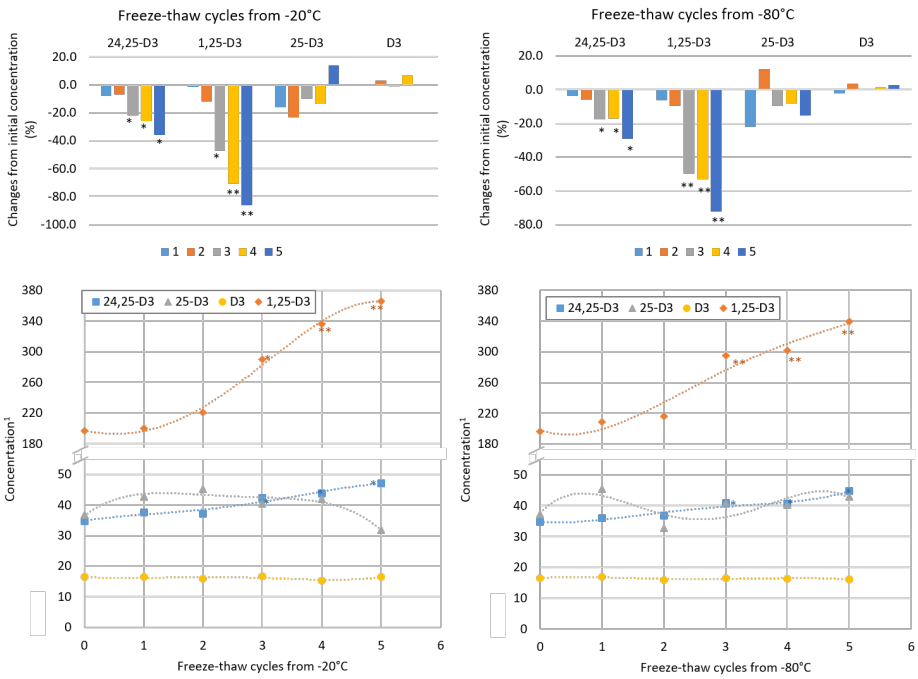

Fig. 2. Evolution of serum concentration of vitamin D_3 and its metabolites with freeze/thaw cycles from –20 °C and –80 °C. ¹The concentration in absolute terms is expressed as nmol/L for all analytes, except for 1,25-dihydroxyvitamin D_3, for which the concentration is expressed as pmol/L.

Concerning the other two metabolites, the concentration of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ significantly decreased after the third thaw cycle in aliquots stored at the two temperatures. Additionally, the decrease in concentration was more relevant at –20 ºC. This effect could be explained by a change in matrix composition after each defrosting cycle since a visible turbidity appears in serum, probably caused by formation of aggregates of various serum components such as

major proteins, which may interfere with the analytical methods. This turbidity increases with the number of freeze/thaw cycles because of the accumulation of these aggregates at the bottom of the sample tubes [22]. These interferents affect in higher extent to the two dihydroxymetabolites, which are the two analytes eluted at shorter times. Therefore, the interferents accumulated by freeze/thaw cycles are injected in the SPE cartridge and coeluted along the first part of the chromatographic gradient.

According to these results, the recommended practice would be to limit the number of freeze/thaw cycles to two, if the target analytes are the two dihydroxymetabolites; whereas $25(OH)D_3$ and vitamin D_3 would be accurately determined after five cycles without any significant effect.

3.3. Sample lyophilization as an alternative for sample storage

Lyophilization is a good alternative to stabilize biological samples due to the removal of water content, which minimizes enzymatic activity. Once the sample is lyophilized, it can be stored at room temperature. This approach was tested in this research as strategy to preserve the concentrations of vitamin D_3 and metabolites for the same period evaluated in the temperature study. For this purpose, lyophilized aliquots of serum were stored at room temperature for 1 and 2 months, after which the target analytes were analyzed and the found concentrations were compared with those found at day 0. The results of this study are presented in Figure 3, which clearly shows that $25(OH)D_3$ did not experience significant changes ($p<0.05$) in concentration after one or two months at room temperature. This result support the stability of this metabolite since its physiological level was not affected by the storage temperature for two months, and this behavior was kept when samples were lyophilized and stored at room temperature. Positively, the same effect was visualized for the two dihydroxy metabolites, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, the concentration of which remained

unaltered (p<0.05) after lyophilization, also for one and two storage months at room temperature.

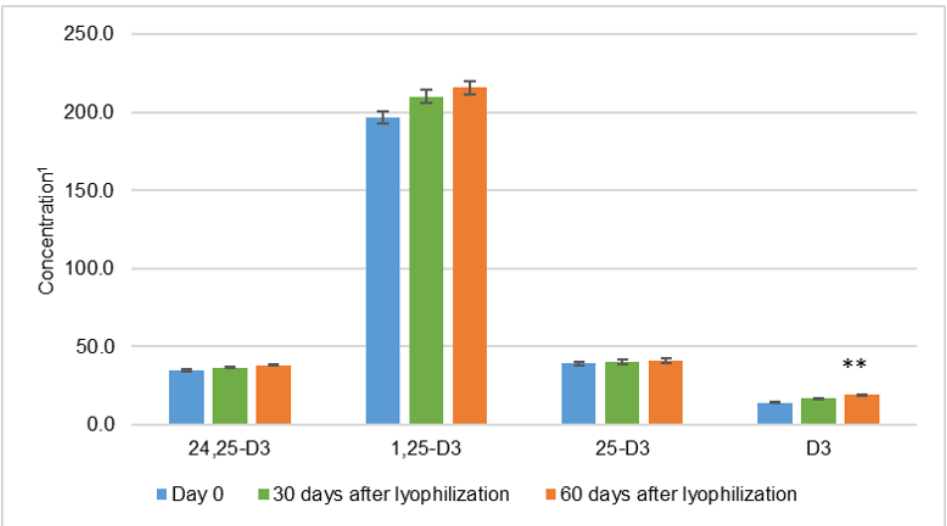

Fig. 3. Concentration of vitamin D_3 and its metabolites at day 0 and after storage for 30 and 60 days at room temperature after sample lyophilization. [1]The concentration is expressed as nmol/L for all analytes, except for 1,25-dihydroxyvitamin D_3, for which the concentration is expressed as pmol/L.

Contrarily, vitamin D_3 experienced decay in its serum concentration in lyophilized samples after two months, although the decrease was not clear when the analysis was carried after one month. Despite vitamin D_3 was less stable than its metabolites, lyophilization can be proposed as an alternative to preserve its concentration in serum at least for one month. On the other hand, the three monitored vitamin D_3 metabolites resulted stable for quantitative analysis in lyophilized samples after two months at room temperature. At short term (two months) the removal of water content seems to play a critical role to guarantee the

stability of vitamin D_3 metabolites at room temperature, considering that the determination of these compounds suffered from significant variations when the aliquots were not frozen

4. Conclusions

Vitamin D is one of the most studied vitamins in human body due to its involvement in several functions. Despite this consideration, the stability of vitamin D_3 metabolites had not been previously evaluated. Thus, the application of a correct strategy for sample storage would lead to more confident results and a reduction of errors associated to sample alteration.

The recommendations in this paper can be followed for the measurement of all vitamin D_3 metabolites in clinical studies, where sensitivity is a critical parameter. This study reports that vitamin D_3 metabolites are stable for two months when serum samples were stored in a freezer at –20 ºC and –80 ºC. The same situation was found for serum subjected to lyophilization, which has demonstrated to be an easy, fast and robust strategy to store at room temperature samples to be used in clinical studies.

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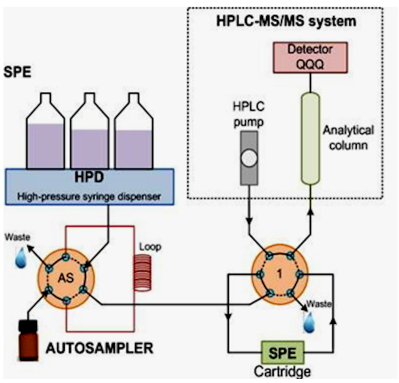
Supplementary material

1. Sample preparation

A serum aliquot of 240 µL was poured in an amber glass vial and spiked with 10 µL of the deuterated working solution —giving the following final concentrations: 25, 25, 5 and 0.3 ng/mL of vitamin D_3-d_3, 25(OH)D_3-d_3, 24,25$(OH)_2D_3$-d_6 and 1,25$(OH)_2D_3$-d_6, respectively—, shaken and located into the autosampler. Supplementary Fig. 1. shows the instrumental arrangement used for analysis of vitamin D and its metabolites. The sample loop was filled with 0.2 mL from the sample vial, which was refrigerated in the autosampler at 6 ºC.



Supplementary Fig. 1. Scheme of the SPE–LC–MS/MS instrumental configuration used in the proposed method.

Then, the serum sample followed the sequence of automatic operations described in Supplementary Table 1. Shortly, the protocol starts by activation of the SPE sorbent with methanol, followed by a conditioning and equilibration step with 25:75 (v/v) ACN–water acidified with 0.7% (v/v) formic acid, the same solution used for sample loading into the cartridge. Under these conditions, the target compounds are retained in the cartridge, which is washed with 30:70 (v/v) ACN–water to remove retained mid-polar interferents. Then, the chromatographic step starts by switching the left clamp valve of the SPE automated station

and putting the cartridge into contact with the initial mobile phase, which also acts as eluent. Elution of the target analytes takes 5 min (longer elution times favor elution of non-polar interferents, which remain retained in the sorbent within the selected interval).

Supplementary Table 1. Steps programmed in the serum SPE protocol.

Step	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				
Start autosampler				Load sample
Solvation 1	10	2	Methanol	
Solvation 2	5	4	Methanol	
Equilibration	3	4	25% ACN–0.7%F	
Equilibration	3	4	25% ACN–0.7%F	
Sample application	3	4	25% ACN–0.7%F	
Wash cartridge	3	0.5	30% ACN	
Elution			Mobile phase	5 min
Purge 1	5	5	Methanol	
Purge 2	5	5	30% ACN	
Purge 3	5	5	Water	
Purge 4	5	5	30% ACN	
Purge 5	5	5	Water	

2. LC–MS/MS analysis

The initial chromatographic mobile phase was 5 mM ammonium formate in 85:15 (v/v) methanol–water at a flow rate of 0.5 mL/min. The temperature of the analytical column compartment was set at 15 ºC. At min 2, a linear gradient was programmed to obtain a 100% 5 mM ammonium formate in methanol at min 5. The final gradient conditions were maintained for 10 min until the end of the chromatographic separation step. The total analysis time was 15 min, 10 min being required for re-establishing and equilibrating the initial conditions. The chromatographic–detection step of a sample and the SPE step of the next sample overlapped, thus improving the analysis frequency.

The eluate from the chromatographic column was monitored by MS/MS in MRM mode. The flow and temperature of the drying gas (N_2) were 9 L/min and 350 ºC, respectively. The nebulizer pressure was 50 psi, and the capillary voltage 4750 V in the positive ionization mode.

Aplicaciones de los métodos desarrollados a la determinación masiva de vitamina D y sus metabolitos

Application of the developed methods to the massive determination of vitamin D and its metabolites

SECTION III

Esta Sección 3 de la Memoria es una prueba irrefutable del excelente funcionamiento del método desarrollado, validado por los resultados que ha proporcionado en su aplicación masiva.

El Capítulo 5 abarca el estudio para la determinación del perfil completo de vitamina D en una cohorte amplia compuesta por 558 controles (mujeres sanas), utilizada en el estudio MCC–Spain. Con los datos que proporcionó la determinación de vitamina D y sus metabolitos en esta población se han tratado de establecer las relaciones entre concentraciones de los metabolitos que a su vez establecen relaciones con factores externos, tales como los antropométricos y los geográficos.

El Capítulo 6 es una compilación de los datos que se obtuvieron en los análisis realizados para diferentes centros de investigación y que se han utilizado para demostrar las hipótesis que propusieron en los correspondientes proyectos para cuyo desarrollo recibieron la correspondiente subvención. Se han publicado artículos científicos basados en los resultados obtenidos tras el tratamiento de parte de los datos de los análisis realizados (ver Anexo II), mientras otra parte de los datos se trata actualmente para obtener la información que soporte futuras publicaciones.

Section III is the irrefutable proof of the excellent performance of the proposed method, assessed by the results it has provided from the massive application.

Thus, Chapter 5 encompasses the study to determine the complete vitamin D profile in a wide cohort composed by 558 controls from an MCC–Spain study. In it, the data of the analysis of vitamin D and its metabolites have been treated to establish the ratios in healthy women that provided relationships between concentrations and external factors, mainly anthropometric or geographical factors.

Chapter 6 is a compilation of the data from the analyses developed for different research centres and used to demonstrate their hypothesis as proposed in their projects. Scientific articles based on the results obtained by handling the data from the analyses have been published (see Annex II), and others are being treated at present aimed at future publications.

Capítulo 5

Vitamin D_3 levels in control women and factors contributing to explain metabolic variations

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Vitamin D_3 levels in control women and factors contributing to explain metabolic variations

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Abstract

The elucidated metabolism of vitamin D_3 in humans has been the support to explain the high involvement of this liposoluble vitamin in physiological functions. Additionally, a wide diversity of clinical studies have associated levels of vitamin D_3 metabolites with several disorders. Despite this knowledge, there are some pending issues related to vitamin D_3 metabolism; the main of them is the controversy on physiological and pathological levels of its metabolites. The association between serum concentrations of vitamin D_3 and its metabolites and several potentially influential factors is analyzed in this study. For this purpose,

558 women were recruited and interviewed in several Spanish provinces before blood sampling. Serum vitamin D_3 and its metabolites were determined using an SPE–LC–MS/MS platform. The concentration range for vitamin D_3 was 1.7–12.2 nmol/L, and it was influenced by body mass index (BMI), waist-to-hip ratio (WHR) and season. 25-hydroxyvitamin D_3 levels were within 4.8–79.8 nmol/L and were related to WHR, season and latitude. The range of 24,25-dihydroxyvitamin D_3, which was between 0.3–8.5 nmol/L, was associated to WHR, season, latitude and calcium intake. Finally, energy intake influenced the 25-hydroxyvitamin D_3/vitamin D_3 ratio.

Keywords: Vitamin D_3; 25-hydroxyvitamin D_3; 1,25-dihydroxyvitamin D_3; 24,25-dihydroxyvitamin D_3; serum; BMI.

1. Introduction

Vitamin D is known as a "prohormone" that has been associated to several body functions in numerous clinical studies [1], and its metabolism is well-known. Vitamin D_3 (cholecalciferol) is synthesized in the skin from pre-vitamin D (7-dehydrocholesterol) by exposure to UV radiation. Then, vitamin D_3 is hydroxylated in the liver producing the circulating form 25-hydroxyvitamin D_3 ($25(OH)D_3$), known as calcidiol, which is the metabolite more frequently determined in clinical studies. Calcidiol is subjected to a second hydroxylation in the kidney and extra renal sites to produce calcitriol, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the fully active form of vitamin D_3 [2,3]. The production of $1,25(OH)_2D_3$ seems to regulate the metabolism of vitamin D_3; thus, when the organism detects enough concentration of calcitriol, calcidiol is transformed in the kidney into $24,25(OH)_2D_3$, one of the major catabolism products of 25(OH)D [4–6]. Despite its clinical relevance, the determination of vitamin D metabolites to provide a complete snapshot of vitamin D status constitutes a challenge owing to their hydrophobic nature, thermal and UV-light instability and similar structure [7].

Most epidemiological studies on vitamin D developed during the last decade have been exclusively devoted to the determination of $25(OH)D_3$, which has been associated to chronic disorders such as diabetes [8], multiple sclerosis [9], cardiovascular diseases [10], asthma [11], several types of cancer —colorectal, breast or prostate cancer [12]—, Parkinson's disease [13] and cystic fibrosis [14]. The main reason to justify selection of $25(OH)D_3$ as the target vitamin D_3 biomarker, despite it is not the active form of vitamin D, is related to the lack of selective and sensitive methods for determination of dihydroxymetabolites [3]. Immunoassay based methods are affected by cross-reactivity with the rest of metabolites, which frequently provide inaccurate results [15]; while mass-spectrometry methods have achieved low detection and quantitation limits for the active metabolite, $1,25(OH)_2D_3$ (pmol/L) [16]. Furthermore, standard reference materials (SRM) for vitamin D_3 metabolites and validation programs such as the

Vitamin D External Quality Assessment Scheme (DEQAS) aid to attain reliable and robust results.

Despite the recent advances in the determination of vitamin D_3 metabolites, the physiological levels of them in human serum are still controversial, and there is no consensus about their optimal concentrations. As an example, there are two different thresholds for $25(OH)D_3$ in human blood established by two USA entities. The Institute of Medicine sets the minimum level of $25(OH)D_3$ at 50 nmol/L [17], and the US Endocrine Society establishes a cut-off at 75 nmol/L [18]. There are no reference concentrations for the other metabolites, although different ranges have been proposed for them. Among the proposed concentration ranges for vitamin D_3 and its metabolites in healthy individuals, Poongkodi *et al.* reported vitamin D_3 values in human adults within the 18–150 nmol/L range [19]; Baecher *et al.* analyzed 50 serum samples from healthy individuals to report 8–165 nmol/L for the vitamin D_3 circulating form $25(OH)D_3$, and 2.8–30 nmol/L for $24,25(OH)_2D_3$ [20]; finally, Hollis *et al.* defined a concentration range from 48 to 168 pmol/L as a normal circulating level for the active form of vitamin D_3 $1,25(OH)_2D_3$ [21].

Due to the controversy about normal levels of vitamin D_3 in blood, recent studies have suggested the possibility of using the concentration ratios between vitamin D metabolites directly connected by a substrate/product relationship [22]. These ratios can provide information about enzymatic activity and could be useful to report a better view of vitamin D status.

This research was aimed at estimating the metabolism of vitamin D_3 in a cohort formed by 558 control women by evaluating the effect of different factors on the concentration of vitamin D_3 and its metabolites. These factors have encompassed age, anthropometric features such as body mass index (BMI) and waist-to-hip ratio (WHR), and seasonal and location factors, among others. Also the ratios between substrate/product pairs will be considered.

2. Material and methods

2.1. Population under study

MCC-Spain is a population-based multicase-control study involving different geographical areas in Spain to identify environmental factors related to the incidence of malignant tumours (http://www.mccspain.org). The design of the study was already described by Castano-Vinyals *et al.* [23]. Briefly, between 2008-2009, more than ten thousand persons aged 20-85 years were interviewed by trained personnel using a computer-assisted program, gathering information on sociodemographic, life-style, reproductive history, hormonal factors, medications and personal and family medical history. The study was approved by the ethical committees of the participating institutions and all participants signed an informed consent for sampling.

This study was focused on a sub-cohort of the MCC-Spain's control group (n=558 women). Participants were randomly selected in primary care clinical centers located in the area of the hospitals where MCC-Spain cases were recruited. Table 1 shows the characteristics of this sub-group of women.

Table 1. Characteristics of the healthy subjects (n=558) studied.

Age and anthropometrics factors							
Age at first birth (years)			*BMI (kg/m2)**		*WHR (cm)***		
24-47	135		underweight	5	0.00-0.77	140	
48-55	131		normal wight	282	0.78-0.83	150	
56-64	134		overweight	192	0.84-0.88	134	
65-85	148		obesity	79	0.89-1.04	134	
Temporal/spatial factors							
Season				*Geographical Region*			
Spring	217			43N	214		
Summer	74			42N	166		
Fall	109			41N	50		
Winter	158			40N	128		
Other factors							
Menopausal status		*Calcium intake (g/day)*		*Calories intake (kcals)*		*Physical activity****	
Postmenopausal	200	<720	126	<1385	126	0 met/week	214
		720-895	126	1385-1700	126	<8 met/week	76
		896-1126	126	1701-2070	126	8-15.9 met/week	78
Premenopausal	358	>1126	126	>2070	126	>=16 met/week	190
		No information	54	No information	54		

*BMI = body mass index **WHT = whaist-to-hip ratio ***met = metabolic equivalent

2.2. Determination of vitamin D_3 and metabolites

The method used for determination of vitamin D_3 and its metabolites was developed beforehand [24]. An automatic solid-phase extraction (SPE) unit on-line connected to a liquid chromatograph–tandem mass spectrometer arrangement (SPE–LC–MS/MS) was used. 200 µL of filtered serum spiked with deuterated standards of each metabolite was prepared for the complete process: sample preparation by SPE followed by liquid chromatography separation with a C18 column and detection by multiple reaction monitoring (MRM) using a triple quadrupole mass detector. Supplementary Information describes the sequence of operations for analysis and includes information about parameters for quantitative determination. Calibration curves for quantitation were obtained by using the ratio between the area of the chromatographic peak from each metabolite and its deuterated standard.

2.3. Statistical analysis

Descriptive analyses of participant's characteristics were performed using absolute figures and means and standard deviations for concentrations of vitamin D_3 and its metabolites. Statistical tests employed to identify significant differences according to the factors evaluated in these women were multifactorial analysis of variance (ANOVA) for categorical factors and Pearson correlation analysis for continuous variables. Both tests were performed using STATGRAPICS Centurion XVI software. A post-hoc Tuckey test was performed for ANOVA analysis. Significance threshold was established at *p*-value below 0.05.

3. Results and discussion

3.1. Serum levels of vitamin D_3 and metabolites

The results obtained from the analysis of vitamin D_3 and its main metabolites in the population under study are presented in Table 2. As can be seen, the most

concentrated metabolite was the circulating form of vitamin D_3, calcidiol, which was detected at an average concentration of 47.3 nmol/L (4.8–79.8 nmol/L). This result is in agreement with previously reported serum levels for calcidiol in healthy individuals: 8–165 nmol/L [20]. This metabolite was almost 7 times more concentrated than its precursor, cholecalciferol, whose average concentration was 7.3 nmol/L (1.7–12.2 nmol/L). These values were below those provided by Poongkodi *et al.*, who reported a range for normal individuals from 18 to 150 nmol/L [19].

Table 2. Vitamin D and its metabolites results obtained in the samples studied after eliminating outliers.

Analytes	Mean	SD	Min.	Max.
Vit D_3 (nmol/L)	7.3	2.1	1.7	12.2
25(OH)D_3 (nmol/L)	47.3	15.4	4.8	79.8
24,25$(OH)_2D_3$ (nmol/L)	3.7	1.8	0.3	8.5
1,25$(OH)_2D_3$ (pmol/L)	113.1	18.5	43.2	148.5
Rates	Mean	SD	Min.	Max.
25(OH)D_3 / Vit D_3	6.8	2.3	0.5	13.1
24,25$(OH)_2D_3$ / 25(OH)D_3	0.08	0.02	0.02	0.14
1,25$(OH)_2D_3$ / 25(OH)D_3	2.7x10-3	1.3x10-3	0.4x10-3	6.3x10-3
24,25$(OH)_2D_3$ / 1,25$(OH)_2D_3$	33.5	17.7	2.6	81.1

*SD =Standard desviation

Concerning the dihydroxymetabolites, the active form, 1,25$(OH)_2D_3$, provided an average concentration of 113.1 pmol/L (43.2–148.5 pmol/L), which was quite similar to the levels provided by Hollis *et al.* (48–168 pmol/L) [21]. On the other hand, the average concentration of 24,25(OH)D_3 was 3.7 nmol/L (0.3–8.5 nmol/L), value only slightly below the normal level previously reported by Baecher *et al.* in healthy human subjects (2.8–30 nmol/L) [20].

Figure 1 shows the distribution of concentrations of the different analytes in the women under study. As can be seen, the levels of vitamin D_3 and 25(OH)D_3 fit a

Gaussian distribution. On the other hand, the distributions for $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were skewed right and left, respectively.

Table 2 also shows the ratio values between pairs of analytes. Thus, the ratio $25(OH)D_3/D_3$ resulted in 6.8±2.3, indicative of the enzymatic activity of 25-hydroxylase in the first hydroxylation step. The conversion rate for production of the two dihydroxymetabolites was expressed by estimation of the $24,25(OH)_2D_3/25(OH)D_3$ and $1,25(OH)_2D_3/25(OH)D_3$ ratios, which were 0.08±0.02 and 0.0027±0.0013, respectively. According to these results, the enzymatic pathway involved in the synthesis of $24,25(OH)_2D_3$ is more active than that for production of $1,25(OH)_2D_3$.

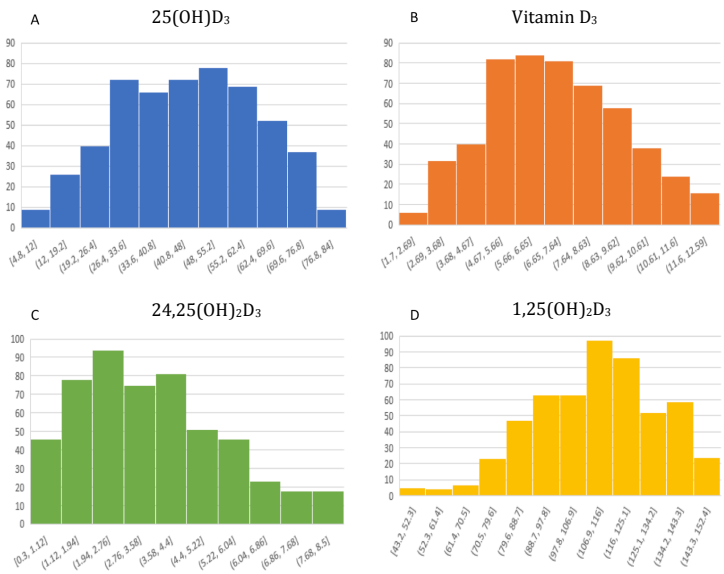

Figure 1. Distribution of concentrations of the different analytes in the cohort under study: A: $25(OH)D_3$ (nmol/L), B: Vitamin D_3 (nmol/L), C: $24,25(OH)_2D_3$ and D: $1,25(OH)_2D_3$.

3.2. Age and anthropometric factors

Vitamin D_3 metabolism has been associated in this research to age and anthropometric factors —BMI and WHR— by application of an ANOVA test. The distribution of individuals according to the three factors is listed in Table 3 that also shows the analytes with a significant variation associated to the given factors. As can be seen, there were not significant differences in the concentration of vitamin D_3 and its metabolites with age.

Table 3. Concentracion of vitamin D and metabolites as a function of age and anthropometrics factors

		Analyte levels			
Age at first birth (years)		*Vitamin D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
24-47	135	7.4 ± 3.4	45.8 ± 20.6	112.3 ± 22.3	3.6 ± 2.8
48-55	131	7.7 ± 3.2	48.7 ±19.5	112.1 ± 24.2	3.8 ± 2.3
56-64	134	7.2 ± 2.7	49.0 ± 19.4	112.6 ± 25.1	4.0 ± 2.2
65-85	148	8.1 ± 2.8	48.8 ± 20.9	117.5 ± 29.2	4.2 ± 3.6
p value		0.63	0.76	0.25	0.59
BMI (kg/m2)		*Vitamin D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
underweight	5	8.2 ± 2.6	63.3 ± 14.8	121.6 ± 13.8	4.1 ± 2.5
normal wight	282	8.4 ± 6.2	49.1 ±19.8	116.4 ± 29.6	4.4 ± 2.9
overweight	192	7.0 ± 2.5	47.8 ± 18.9	111.8 ± 23.0	4.0 ± 3.4
obesity	79	6.2 ± 2.5	49.3 ± 22.4	109.4 ± 28.2	3.1 ± 2.0
p value		0.0003	0.95	0.16	0.06
WHR (cm)		*Vitamin D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
0.00-0.77	140	8.5 ± 3.5	49.8 ± 21.1	110.3 ± 22.7	4.1 ± 3.5
0.78-0.83	150	7.6 ± 2.7	50.7 ± 21.3	114.4 ± 22.7	4.3 ± 2.8
0.84-0.88	134	7.8 ± 8.2	47.0 ± 17.4	116.8 ± 20.1	3.9 ± 2.4
0.89-1.04	134	6.5 ± 2.3	44.2 ± 18.9	113.5 ± 22.9	3.4 ± 2.1
p value		0.001	0.02	0.3	0.04

Concerning the BMI, only the level of vitamin D_3 was significantly altered (*p*-value 0.0003) in individuals grouped in underweight (<18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2) and obese (≥30.0 kg/m^2)

categories. Figure 2 shows the distribution of vitamin D_3 levels that decreased with the increase of BMI. This trend has been previously described by Avtanski *et al.* [25].

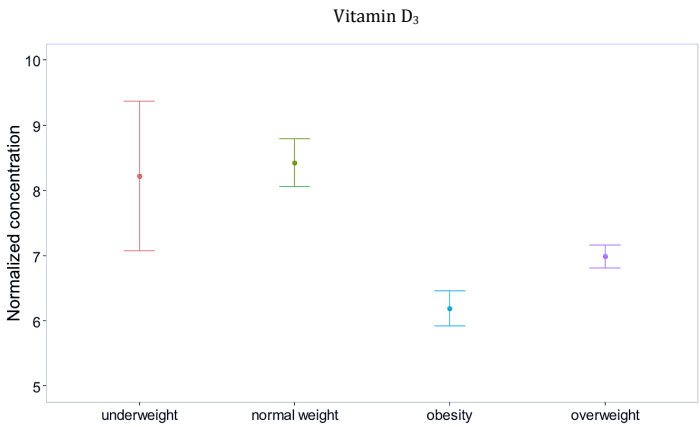

Figure 2. Distribution of Vitamin D_3 concentrations for each class of BMI (underweight, normal weight, overweight and obesity).

The WHR was a better parameter to explain the variability of vitamin D_3 metabolism since vitamin D_3 (*p*-value 0.001) and two metabolites, particularly, $25(OH)D_3$ (*p*-value 0.02) and $24,25(OH)_2D_3$ (*p*-value 0.04), reported significant differences according to WHR. The three compounds were characterized by the same trend, giving a decrease of their concentration when the WHR increased (Figure 3).

The results provided by BMI and WHR suggest that obesity is associated with vitamin D_3 deficiency through a decrease of metabolism. Vitamin D is liposoluble and, thus, it is mainly stored in the adipose tissue that serves as an endogenous source of vitamin D [26]. For this reason, individuals with high BMI and WHI present levels of vitamin D in blood lower than normal weight individuals. In fact,

different authors have shown that vitamin D levels increased in blood when there was a body weight loss [25]. One other reason explaining the association of obesity and vitamin D levels is the suppressed expression of adipose 25-hydroxylase [25] in obese individuals, a key enzyme for vitamin D synthesis regulation.

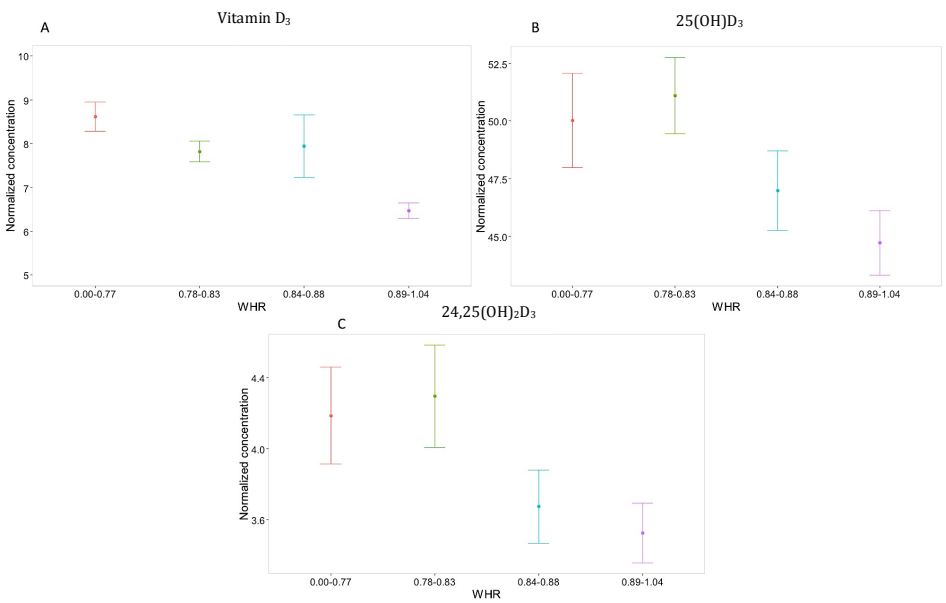

Figure 3. Distribution of A: Vitamin D_3, B: $25(OH)D_3$ and C: $24,25(OH)_2D_3$ concentrations for each level of WHR.

The active form $1,25(OH)_2D_3$ did not report significant differences as a function of BMI and WHR. The result could be justified because the production of this metabolite takes place in different tissues (bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells, several and the parathyroid gland), where the enzyme 1α-hydroxylase is expressed [27]. So, this variability in the production tissues could support the lack of correlation.

3.3. Seasonal and spatial factors

The influence of the seasonal period on vitamin D_3 levels was studied by considering vitamin D_3 and its three metabolites. For this purpose, the samples were organized according to the seasonal period in which they were extracted: fall, winter, spring and summer. Table 4 lists the distribution of samples according to the seasonal period and levels found for all analytes.

Table 4. Concentrations of vitamin D and metabolites as a function of temporal/spatial factors.

		Analyte levels			
Season		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
Spring	217	7.2 ± 2.5	44.5 ± 19.3	113.8 ± 22.0	3.3 ± 2.1
Summer	74	8.2 ± 3.4	54.8 ±19.7	119.6 ± 23.6	5.2 ± 2.8
Fall	109	7.6 ± 2.7	51.4 ± 19.6	110.6 ± 25.3	4.4 ± 2.6
Winter	158	7.4 ± 2.8	47.4 ± 20.1	113.0 ± 22.3	3.7 ± 3.4
p value		0.003	0.0003	0.29	0.0001
Geographical Region		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
43N	214	7.0 ± 3.6	48.9 ± 19.9	112.0 ± 21.7	3.8 ± 2.4
42N	166	7.9 ± 2.4	43.9 ±18.0	116.4 ± 21.0	3.7 ± 2.3
41N	50	7.6 ± 3.0	46.0 ± 22.8	108.4 ± 26.7	4.9 ± 3.3
40N	128	8.5 ± 2.1	52.5 ± 20.3	116.3 ± 24.4	3.7 ± 3.6
p value		0.053	0.003	0.26	0.03

The ANOVA test revealed that vitamin D_3 (*p*-value 0.003), 25(OH)D_3 (*p*-value 0.0003) and 24,25(OH)$_2D_3$, (*p*-value <0.0001) provided the same variability trend, and their levels decreased by following the sequence summer>fall>winter>spring, as Figure 4 shows. This trend corroborates that the season is one of the main factors affecting vitamin D_3 serum levels, including its metabolites. Additionally, the results reported in summer were logical considering that the exposure to UV irradiation is clearly higher along this season. However, a memory effect seems to exist that would explain why vitamin D levels in fall and winter were above the concentrations found in spring.

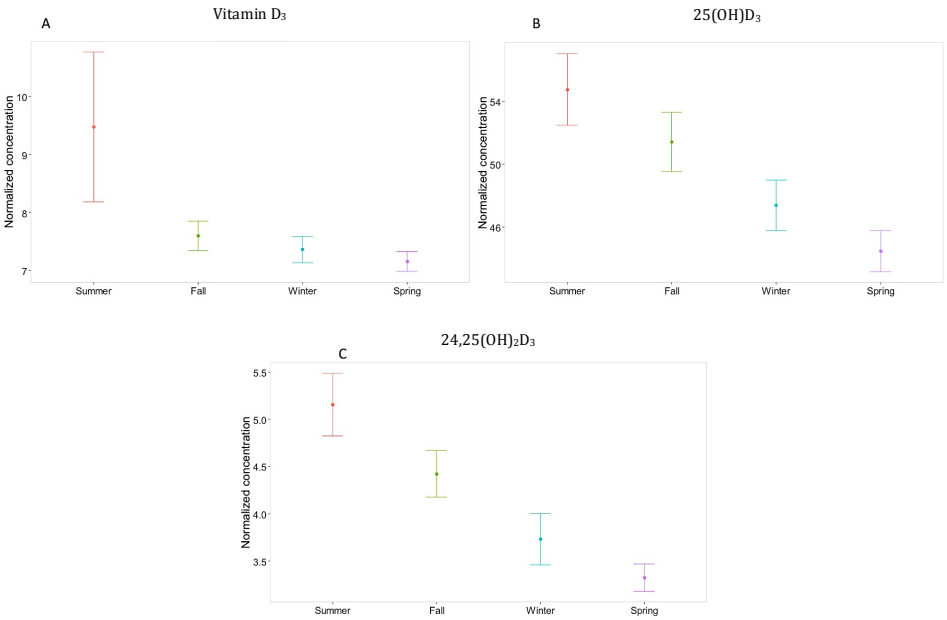

Figure 4. Distribution of season period for A: Vitamin D_3, B: $25(OH)D_3$ and C: $24,25(OH)_2D_3$.

A spatial factor was defined according to the geographical origin of samples by considering four different latitudes in Spain: 43N, 42N, 41N and 40N, which are shown in Figure 5. Table 4 shows the distribution of samples according to this spatial factor and levels of vitamin D_3 and its metabolites in the samples corresponding to each group. The ANOVA test pointed out that the concentrations of $25(OH)D_3$ (p-value 0.003) and $24,25(OH)_2D_3$ (p-value 0.03) varied significantly as a function of the latitude. In the case of $25(OH)D_3$ the post-hoc analysis based on the Tuckey test allowed identifying differences between latitudes 40N and 42N, and between 42N and 43N. As Figure 5 shows, women living in a latitude 42N presented lower levels of $25(OH)D_3$ metabolite. The levels of $24,25(OH)_2D_3$ also

experienced significant variability according to the latitude, with the group from latitude 41N clearly showing higher concentration than those from the rest of the geographical areas (Figure 5).

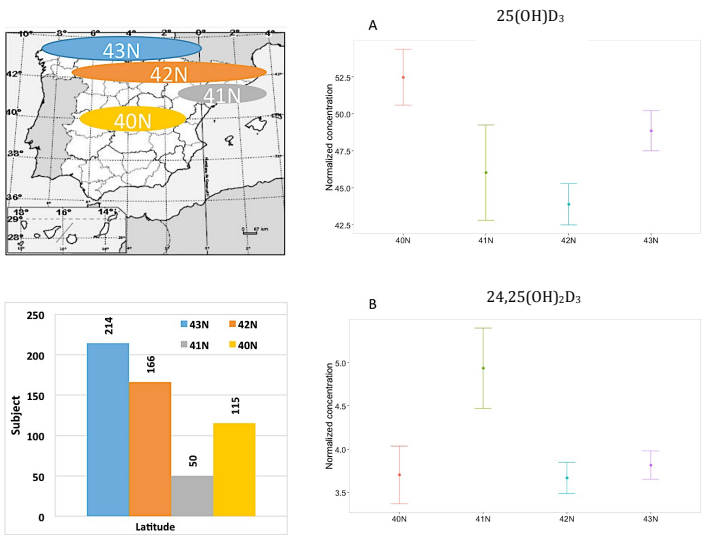

Figure 5. Geographical latitudes studied, classification of the subject by latitude and distribution of concentration for A: $25(OH)D_3$ and B: $24,25(OH)_2D_3$.

Higher levels found for $25(OH)D_3$ in latitudes 40N and 43N could be explained because the area that fits latitude 40N is located further South than the rest of the areas, and the area pertaining to latitude 43N is a coastal area. Exposure to UV irradiation should be higher in these areas as compared to other latitudes. On the other hand, $24,25(OH)_2D_3$ was found at higher concentrations in women from latitude 41N, which is another coastal area in the northeast of Spain.

As occurred with anthropometric factors, no association was found between season and latitude with levels of $1,25(OH)_2D_3$.

3.4. Influence of other factors on the levels of vitamin D_3 and its metabolites

An additional study was executed to find the influence of other factors —*viz*. physical activity in the last 5 years (metabolic equivalent (met)/week), menopause status, calcium intake (g/day), and energy intake (kcal)— on vitamin D levels in blood (Table 5).

Table 5. Concentration of vitamin D and metabolites as a function of different factors.

		Analyte levels			
Menopausal status		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
Postmenopausal	200	7.5 ± 2.8	49.1 ± 19.5	113.0 ± 23.1	4.1 ± 2.8
Premenopausal	358	7.4 ± 3.2	46.1 ± 20.6	112.5 ± 22.6	3.6 ± 2.7
p value		0.45	0.09	0.47	0.07
Calcium intake		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
0	126	7.0 ± 2.8	44.7 ± 16.7	110.0 ± 21.6	3.3 ± 2.0
1	126	8.1 ± 2.3	47.5 ±21.9	114.7 ± 20.3	4.0 ± 2.6
2	126	7.4 ± 2.9	49.9 ± 19.1	115.4 ± 23.2	4.2 ± 3.6
3	126	8.2 ± 3.6	50.5 ± 20.6	114.0 ± 24.2	4.1 ± 3.6
p value		0.14	0.08	0.53	0.02
Calories intake		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
0	126	7.3 ± 2.9	48.1 ± 21.1	110.6 ± 20.9	3.8 ± 2.5
1	126	7.8 ± 2.2	46.3 ±18.2	116.9 ± 23.8	3.7 ± 3.6
2	126	7.4 ± 2.8	51.0 ± 18.8	110.8 ± 21.9	4.2 ± 2.2
3	126	8.2 ± 3.7	47.2 ± 20.7	115.9 ± 24.8	3.9 ± 2.7
p value		0.52	0.26	0.27	0.45
Physical activity		*Vit D_3 (nmol/L)*	*25(OH)D_3 (nmol/L)*	*1,25(OH)$_2D_3$ (pmol/L)*	*24,25(OH)$_2D_3$ (nmol/L)*
0 mets/sem (media año)	214	7.2 ± 2.8	46.4 ± 19.9	112.6 ± 24.2	3.6 ± 2.5
<8 mets/sem (media año)	76	7.5 ± 3.8	49.1 ± 18.6	112.4 ± 24.0	4.0 ± 2.4
8-15.9 mets/sem (media año)	78	7.9 ± 2.9	49.0 ± 17.6	113.0 ± 23.5	3.7 ± 2.1
>=16 mets/sem (media año)	190	8.0 ± 2.5	49.1 ± 21.3	115.8 ± 28.2	4.3 ± 3.4
p value		0.29	0.25	0.58	0.07

After statistical evaluation by ANOVA test, only calcium and energy intake contributed to explain significantly the levels of vitamin D. One of the

dihydroxymetabolites, $24,25(OH)_2D_3$, was significantly associated with calcium intake (*p*-value 0.02). The post-hoc test allowed identifying significant differences between group 0 (<720 g/day) and groups 1 (720–895 g/day), 2 (896–1126 g/day) and 3 (>1126 g/day). Thus, Figure 6A illustrates the increased concentration of this metabolite with the increased intake of calcium, suggesting a correlation effect between calcium levels and the formation of $24,25(OH)_2D_3$, the main product of vitamin D_3 catabolism. There are not published studies relating the intake of calcium to the levels of this dihydroxymetabolite. Nevertheless, some studies have already suggested that calcium intake could influence $25(OH)D_3$ metabolic consumption [28–32], and also seems to increase the half-life of $25(OH)D_3$ [33]. These two effects could affect the concentration of related metabolites.

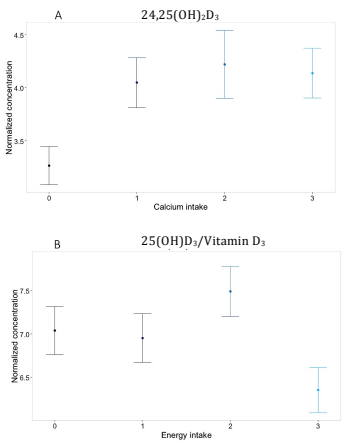

Figure 6. Distribution for concentrations of A: levels of $24,25(OH)_2D_3$ taking calcium and B: ratios of $25(OH)D_3$/Vitamin D_3 relationated with energy intake by the cohort.

Concerning the energy intake, the analysis revealed significant differences of the ratio between the circulating form and its precursor, $25(OH)D_3$/vitamin D_3 (*p*-

value of 0.03). Concretely, Figure 6.B shows that the ratio increased with the energy intake up to 2070 kcal, while higher energy intakes led to a decrease of this ratio. Attending to these results, there is an activation/inhibition effect of the vitamin D_3 metabolism ascribed to energy intake.

4. Conclusions

Vitamin D_3 and its most important metabolites have been analyzed in serum from 558 control women to evaluate the incidence of several factors on vitamin D metabolism. The WHR factor especially affected the variability in the levels of vitamin D_3, $25(OH)D_3$ and $24,25(OH)_2D_3$, which are involved in the most favored pathway in terms of concentration. A similar effect was observed for the seasonal period of the year, which proves the variability of vitamin D_3 metabolism according to exposure to UV irradiation. Calcium and energy intake also contributed to explain the observed differences in the concentration of vitamin D_3 and metabolites. Attending to these results, it is worth considering all these factors in intervention studies dealing with monitoring the activity of vitamin D_3 metabolism. Other remarkable aspect is the complexity of setting cut-off values for vitamin D_3 concentration due to the high influence of these factors.

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Capítulo 6

Application of the developed methods to clinical studies coordinated by other research centers

Application of the developed methods to clinical studies coordinated by other research centers

1. Introduction

The two new analytical platforms in Chapters 1 and 2, and the knowledge acquired in Chapters 3 and 4 for determination of vitamin D and its metabolites were applied to clinical studies coordinated by other national and international research centers. A total number of 2890 human serum samples were analyzed by the developed methods. This large amount of samples proves the suitability and robustness of the developed platforms. Furthermore, the reported results have been used [1] or are being currently processed to write research articles for publication in high-impact factor journals. The analysis of this number of samples was possible thanks to the almost full automation of the method, its high frequency of analysis, and the short time required for sample handling before analysis.

A big strength of the methods proposed in this Thesis was the support of a reliable external validation program for determination of vitamin D metabolites. This program, vitamin D External Quality Assurance Scheme (DEQAS), allows validating analytical methods for determination of total $25(OH)D_3$ and total $1,25(OH)_2D_3$. In the last three editions (2015/2016, 2016/2017 and 2017/2018), the research group obtained the proficiency certificate for determination of $25(OH)D_3$, since a number of samples above 75% for each edition reported results with a variability below 25% as compared to the target value. The proficiency certificate was not obtained for $1,25(OH)_2D_3$, and two possible reasons can explain this circumstance. Firstly, the reference concentration value for each sample is the All-Laboratory Trimmed Mean (ALTM), which is less reliable than the $25(OH)D_3$ concentration certified by the NIST for each external sample. Additionally, DEQAS samples are sent without refrigerated conditions for transport and, as described in Chapter 4, sample storage at room temperature is not the suited strategy prior to

determination of 1,25(OH)$_2$D$_3$; on the contrary, 25(OH)D$_3$ levels are not affected by this transport conditions.

Three national and international research centers have demanded for the analysis of vitamin D metabolism in different clinical studies carried out along the development of this Thesis. The demands gave place to research contracts with the corresponding institution and, in some cases, collaborations were set to prepare publications based on the obtained results. In all cases the principal aim of the analyses was to establish associations between vitamin D concentrations and different disorders as multiple sclerosis, breast cancer, or physiological states such as menopause.

2. Multiple sclerosis study

A collaboration was established with Dr. Dalla Costa from Inflammatory Central Nervous System Disorders Unit of Ospedale San Raffaele (Milan, Italy). The main aim of this research was to determine the levels of vitamin D and metabolites in patients with clinically isolated syndromes and to associate this with the risk from multiple sclerosis. For this purpose, two batches of samples were analyzed with the two different methods.

The first batch was constituted by 114 serum samples from Italian patients diagnosed with clinical isolated syndrome (CIS) and treated in the Ospedale San Raffaele between 2000 and 2012. This batch of samples was analyzed with the platform described in Chapter 2 (SPE–2DLC–MS/MS); therefore, vitamin D$_3$ and its metabolites were determined, with discrimination of the C3-epi-25(OH)D$_3$ form. The results obtained from this batch are listed in Table 1.

These results were presented in the European Academy of Neurology Congress held in Copenhagen, 2016 [2]. The principal conclusion of this first study was that high 25(OH)D$_3$ levels were associated with a low risk of suffering multiple sclerosis.

A total of 52% of CIS patients reported vitamin D deficiency, by setting the cut-off level at 50 nmol/L. During follow-up (median: 7.17 years), 55 patients developed clinically definite multiple sclerosis (CDMS). Among patients with medium or high levels of $25(OH)D_3$, two clusters were identified, where the cluster with a significant lower production of active and inactive metabolites (low CYP27 activity) had a significantly higher risk of MS.

Table 1. Results from the collaboration with Ospedale San Raffaele.

Batch	Samples	Vitamin D_3 (nmol/L)	$25(OH)D_3$ (nmol/L)	C3-epi-$25(OH)D_3$ (nmol/L)	$24,25(OH)_2D_3$ (nmol/L)	$1,25(OH)_2D_3$ (pmol/L)
1	114	5.5 ± 5.1	29.6 ± 12.9	0.8 ± 0.7	2.3 ± 1.3	107.6 ± 19.2
2	200	4.3 ± 2.8	50.3 ± 22.3	–	8.2 ± 5.5	100.7 ± 21.2

The second batch was composed by 200 serum samples that were analyzed with the platform described in Chapter 1. Therefore, no discrimination was done between the $25(OH)D_3$ epimers, as shown in Table 1.

The results of this second batch are being treated by the research group of Dr. Dalla Costa searching for correlation between basal vitamin D levels and disease activity in multiple sclerosis patients treated with fingolimod.

3. MCC-Spain: Multi-case control cancer study in Spain

The involvement of the FQM-227 research group in this project resulted from the collaboration established with Dr. Marina Pollán from the National Center for Epidemiology in Carlos III Institute of Health (Madrid, Spain) and member of CIBERESP (Consortium for Biomedical Research in Epidemiology & Public Health). The collaboration with Dr. Pollán involved the association of the levels of vitamin

D_3 and metabolites levels with breast cancer risk by pathological subtype, stage at diagnosis and specific breast cancer risk factors.

The cohort of this study included 1104 serum samples from women enrolled in a multicase-control study in Spain (MCC-Spain) [3], with 546 histologically confirmed breast cancer cases and 558 control individuals. In this study, the samples were analyzed for quantitative determination of vitamin D_3 and metabolites by the platform SPE–LC–MS/MS described in Chapter 1. A summary of the results from the analysis of the samples is shown in Table 2. These results have been used by Lope *et al.* [1] to evaluate statistically the association among $25(OH)D_3$ levels and breast cancer risk, the dose-response shape of this relationship and possible differences in this association among menopausal status, pathologic subtype and stage at diagnosis in a sub-sample of untreated breast cancer cases and population-based controls.

Table 2. Results from all analyzed samples belonging to the MCC-Spain study.

Samples	Vitamin D_3 (nmol/L)	$25(OH)D_3$ (nmol/L)	$24,25(OH)_2D_3$ (nmol/L)	$1,25(OH)_2D_3$ (pmol/L)
1104	7.2 ± 4.0	45.8 ± 21.2	3.8 ± 2.7	113.0 ± 28.0

The obtained results have been published in Journal of Steroid Biochemistry and Molecular Biology [1]. The principal conclusion was an inverse association between $25(OH)D_3$ serum levels and breast cancer risk, which was more pronounced in triple negative tumors. The authors finally concluded that public health and clinical strategies aimed at improving vitamin D levels would be desirable, taking into account the high proportion of women with inadequate concentration of 25(OH)D.

4. The DDM-Madrid study

A second collaboration with Dr. Marina Pollán from National Center for Epidemiology in Carlos III Institute of Health in the frame of the DDM-Madrid study was developed, which also has given place to an article in production progress of which the PhD student is an author [4]. In this study 1472 serum samples from premenopausal women (39–50 years old, recruited between June 2013 and May 2015) were analyzed. The aim of this research was to evaluate the effect of vitamin D_3 and its principal metabolites on the mammographic density (MD) in premenopausal women. The method described in Chapter 1 was used to determine the concentration of the target analytes in this batch, the results of which are listed in Table 3.

Table 3. Results from all samples analyzed in the menopausal-women study.

Samples	Vitamin D_3 (nmol/L)	$25(OH)D_3$ (nmol/L)	$24,25(OH)_2D_3$ (nmol/L)	$1,25(OH)_2D_3$ (pmol/L)
1472	6.6 ± 2.7	49.2 ± 18.9	3.3 ± 1.5	111.1 ± 26.1

The results showed that the MD in women with sufficient serum 25(OH)D levels (>75 nmol/L) was slightly lower than the MD of women with deficient levels, being this protective effect more accentuated among nulliparous women. The protective effect of vitamin D on MD and the high prevalence of vitamin D insufficiency in the cohort population were proved; therefore, to enhance vitamin D levels should be proposed as an effective way to prevent postmenopausal increased MD, which can derive in breast cancer.

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DISCUSIÓN DE LOS RESULTADOS

DISCUSSION OF THE RESULTS

Las normas vigentes en la Universidad de Córdoba sobre el modelo de Tesis Doctoral en el formato de compilación de artículos (publicados o próximos a su publicación) hacen obligatoria una sección para la discusión conjunta de los resultados.

La investigación en esta Tesis Doctoral, dedicada completamente a la vitamina D y sus principales metabolitos en humanos, se puede dividir, en función de los objetivos, en tres secciones que coinciden con las tres etapas principales de la investigación analítica: desarrollo de métodos, preparación de la muestra y aplicación a muestras reales.

Por lo tanto, la Sección I está enfocada al desarrollo de nuevos métodos, Capítulo 1 y 2, para aumentar el número de metabolitos de la vitamina D determinados en un solo análisis de forma automática, minimizando así el error humano y mejorando la sensibilidad y la selectividad utilizando estándares internos deuterados, diferentes configuraciones de columnas para la separación analítica y utilizando la espectrometría de masas para la determinación.

La segunda sección trata sobre una etapa clave y poco estudiada en el proceso analítico para determinar la vitamina D: la preparación de la muestra. En esta sección se evaluaron dos muestras de sangre (suero y plasma) para comparar la determinación de los metabolitos de la vitamina D en las dos matrices biológicas. También se compararon dos formas de preparación de la muestra (precipitación de proteínas y extracción en fase sólida en línea), todo lo cual se recoge en el Capítulo 3. El Capítulo 4 abarca un estudio de estabilidad de la muestra, en el que se estableció la temperatura más adecuada para conservar la muestra y el número máximo de ciclos de congelación/descongelación a los que puede someterse la muestra para cada uno de los compuestos diana. Finalmente, se propuso un nuevo modo de conservación de la muestra a temperatura ambiente basado en su liofilización.

En la Sección III, y como evaluación de los resultados de las dos secciones anteriores, se llevó a cabo el análisis masivo de muestras de suero de varias cohortes de pacientes. El estudio MCC (Capítulo 5) se centró en la determinación de los metabolitos de la vitamina D en mujeres sanas y en el análisis estadístico de los resultados. Los otros estudios, que abarcaron muestras de niños con esclerosis múltiple en niños, mujeres con cáncer de mama y mujeres osteoporóticas, se han recopilado en el Capítulo 6, para mostrar la aplicabilidad de la plataforma analítica desarrollada en esta Tesis. El Anexo 4 contiene una publicación basada en los resultados obtenidos mediante el análisis de muestras de una de estas cohortes masivas.

Sección I: Mejora de las plataformas analíticas para la determinación de vitamina D y sus metabolitos

El creciente número de enfermedades relacionadas con la vitamina D y/o sus principales metabolitos parece desempeñar un papel crucial en el aumento del interés por el desarrollo de nuevos métodos para la determinación de estos compuestos. La complejidad del análisis de estos metabolitos se debe a su similitud estructural, naturaleza hidrofóbica e inestabilidad térmica y a la radiación UV. Los métodos recientes para la determinación de los metabolitos principales van desde inmunoensayo (ELISA, RIA, CPB) a los basados en la separación mediante cromatografía, tanto de líquidos como de gases, y en la determinación mediante absorción molecular UV visible, ionización en llama o espectrometría de masas (MS).

Los métodos basados en cromatografía de líquidos–espectrometría de masas en tándem (LC–MS/MS) se consideran actualmente "*gold standard*", pero las bajas concentraciones de los analitos en matrices biológicas obligan a realizar etapas de preconcentración y limpieza, para los cuales la SPE automatizada constituye una

excelente herramienta que facilita y acelera la preparación de la muestra. Además, este dispositivo se puede acoplar en línea a los equipos de LC, lo que mejora la robustez y la velocidad del análisis.

Por otro lado, la determinación de 3-epi-25(OH)D_3 y 25(OH)D_3 es compleja, ya que ambos metabolitos tienen una composición elemental idéntica, y las transiciones *MRM* para su cuantificación también lo son. No obstante, su estructura es diferente, lo que lleva a una sobreestimación de la concentración de 25(OH)D_3 en la mayoría de los métodos LC–MS/MS, que no pueden diferenciar los epímeros co-eluidos. El diseño de una plataforma cromatográfica bidimensional ha permitido la separación de ambos analitos, además de la determinación de estos isómeros, la de otros metabolitos cruciales de la vitamina D.

Los resultados de esta investigación constituyen los Capítulos 1 y 2 y se discuten a continuación.

El primer estudio tuvo como objetivo optimizar y validar una plataforma automatizada SPE–LC–MS/MS cuyas características analíticas permitían la determinación de la vitamina D y sus principales metabolitos. El método se dirigió a las vitaminas D_2 y D_3, los monohidroximetabolitos y, principalmente, a los dihidroximetabolitos de las dos formas de vitamina D: 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ y 24,25$(OH)_2D_3$. La inclusión de la vitamina D_2 y sus metabolitos en los métodos analíticos desarrollados es de particular interés cuando esta forma se administra como suplemento, una práctica común en Estados Unidos y en el norte de Europa.

La optimización de la etapa de SPE fue crucial teniendo en cuenta los bajos niveles a los que se encuentran los metabolitos de la vitamina D en sangre; por lo que esta etapa se centró en la preconcentración de los analitos y la eliminación de interferentes. Se estudiaron cartuchos de dos sorbentes (C18 y C8) para seleccionar el más apropiado. Aunque el de C18 mostró buenos resultados para la

vitamina D, debido a su mayor interacción lipofílica con este analito, se seleccionó C8, ya que este sorbente fue el mejor para los mono- y dihidroximetabolitos.

Se utilizó el estándar deuterado correspondiente para cada analito, lo que tras la separación cromatográfica permitió su determinación inequívoca. Además, los iones producto utilizados en la monitorización fueron iguales para cada metabolito y su correspondiente estándar deuterado.

Las características analíticas del método hicieron que esta plataforma fuera muy apropiada para determinar los metabolitos de interés en muestras de suero, mostrando buenos límites de detección y de cuantificación (LOD y LOQ, espectivamente). El método proporcionó una variabilidad en el mismo día entre 1.5 y 11.5% y una variabilidad entre días inferior al 13%, ambas expresadas como desviación estándar relativa (RSD). El factor de recuperación fue cercano al 100% para di- y monohidroximetabolitos, y alrededor del 60% para las vitaminas D_3 y D_2; lo que se explica por la baja eficiencia de extracción del sorbente C8 para las vitaminas, menos polares que sus metabolitos. Finalmente, se obtuvo una excelente precisión para el método, entre el 89.3 y el 93.6%.

La validación se realizó mediante evaluación externa de la calidad (DEQAS), utilizando 10 muestras para determinar el total de 25(OH)D y otras 10 para el total de $1,25(OH)_2D$. En ambos casos los resultados estuvieron dentro de los límites impuestos por DEQAS.

El siguiente paso fue desarrollar un nuevo método analítico también para los metabolitos de la vitamina D en suero humano, que incluyese en este caso la forma 3-epi-$25(OH)D_3$. El método involucró SPE en línea acoplada con a la separación cromatográfica bidimensional (2DLC) y a la detección de MS/MS. Se utilizaron una columna C18 y otra de pentafluorofenilo (PFP) para la separación de los dos epímeros de $25(OH)D_3$ sin que se viera afectada la sensibilidad y la resolución para los otros metabolitos principales de la vitamina D.

Se realizó una primera comparación entre las dos columnas (PFP y C18) para obtener una buena separación previa a la determinación de todos los analitos. La columna PFP permitió la separación entre 25(OH)D_2, 25(OH)D_3 y 3-epi-25(OH)D_3, pero con una pobre sensibilidad para los dihidroximetabolitos; mientras que la columna C18 proporcionó una sensibilidad suficiente para todos los analitos, pero la separación del epímero de los otros mono-hidroximetabolitos fue imposible. Por esta razón, se desarrolló una configuración 2DLC para combinar las buenas propiedades de cada columna en una sola plataforma. Primero, los dihidroximetabolitos se separaron en la columna C18, seguido de la separación de los monohidroximetabolitos por la columna PFP y, finalmente, las dos formas de la vitamina D se separaron en la columna C18. Todo el proceso se diseñó para su control automático, de forma que todo el eluato de la SPE pasara a la primera de las columnas cromatográficas; lo que finalmente dio lugar a que el espectrómetro de masas proporcionara un excelente cromatograma.

La sensibilidad proporcionada por la plataforma 2DLC fue bastante similar a la reportada en el Capítulo 1, en el que el enfoque era parecido, pero utilizando solo una columna para la separación. Para el nuevo analito, el epímero, los valores de LOD y LOQ fueron muy similares a los de los otros monohidroximetabolitos y permitieron su determinación en suero humano. También la precisión proporcionada por esta plataforma fue similar a la del Capítulo 1: variabilidad entre días inferior al 11.6% y variabilidad en el mismo día inferior al 7.2% para todos los analitos (valores siempre expresados como RSD). La precisión para 3-epi-25(OH)D_3 fue ligeramente más baja que para la forma 25(OH)D_3, para la cual la precisión no se vio afectada por la separación 2DLC.

Los factores de recuperación fueron alrededor del 100% para los dihidroximetabolitos y superiores al 95,2% para los monohidroximetabolitos, incluido el 3-epímero. Por otro lado, y como en el Capítulo 1, el factor de recuperación disminuyó a 59.1 y 60.2 para las vitaminas D_2 y D_3, respectivamente. La precisión del método y los posibles efectos de la matriz oscilaron entre 83.3 y

96.1, y 95.5% para el epímero C3; valores de sensibilidad adecuados para su cuantificación en suero humano.

Finalmente, la validación externa abarcó dos formas: la de los monohidroximetabolitos por un material de referencia de la NIST, y la validación externa DEQAS como en el Capítulo 1. Con el material de referencia de la NIST se realizó un análisis por triplicado para cada uno de los cuatro niveles de concentración suministrado, y el porcentaje de sesgo en comparación con los valores de referencia varió entre el 2.0 y el 8.5%, corroborando así la precisión del enfoque propuesto para el análisis de los tres monohidroximetabolitos. Se utilizaron cinco muestras de DEQAS para validar externamente los dihidroximetabolitos de acuerdo con los límites establecidos por DEQAS.

Sección II: Estudios sobre la preparación y estabilidad de la muestra para la determinación de vitamina D y sus metabolitos

Esta sección se centra en la etapa de preparación de la muestra (Capítulo 3) para la determinación de los principales metabolitos de la vitamina D, así como en diferentes estrategias de conservación de las muestras de suero en estudios clínicos, para evitar problemas de degradación (Capítulo 4). En ambos estudios el objetivo principal fue mejorar la sensibilidad del método, considerando la baja concentración de los metabolitos diana en la sangre humana (niveles de nmol/L o pmol/L).

La bibliografía recoge las diferentes muestras biológicas que se han utilizado para el análisis de la vitamina D y sus metabolitos (p. ej., saliva, líquido cefalorraquídeo o sangre seca), pero el suero y el plasma persisten como las principales matrices biológicas para la determinación de estos compuestos, ya que ambos pueden obtenerse fácilmente y contienen los principales metabolitos a niveles cuantificables. Los protocolos de preparación de muestras basados en suero o plasma se han utilizado ampliamente, pero nunca se han comparado entre ellos.

Con estos precedentes, se estudiaron dos estrategias para la preparación de muestras de suero y plasma (precipitación de proteínas y SPE en línea), que se optimizaron para cada matriz biológica.

Otra variable crítica debido a la naturaleza de los metabolitos de la vitamina D es su estabilidad en muestras clínicas. Éste es un aspecto clave para una evaluación fiable de los resultados cuantitativos en la investigación epidemiológica. El comportamiento de los analitos objetivo desde la extracción de sangre hasta su determinación tiene una relevancia crítica. El tiempo y las condiciones a las que se deben almacenar las muestras fueron aspectos clave a estudiar en profundidad, ya que la estabilidad de los metabolitos de la vitamina D en sangre no se había estudiado suficientemente hasta el momento. De hecho, a pesar de la inestabilidad en sangre de los metabolitos de la vitamina D, solo se había estudiado anteriormente la del metabolito $25(OH)D_3$.

Los resultados obtenidos en estos estudios se discuten en detalle a continuación.

La investigación sobre la influencia de la muestra de sangre utilizada (suero o plasma) para el análisis cuantitativo de los analitos diana implicó: (i) el tipo de muestra, suero o plasma; (ii) la presencia de gel en los tubos para la extracción de sangre (utilizada para favorecer la separación del suero o plasma de las células sanguíneas); y (iii) la evaluación de la precipitación de proteínas o SPE automatizada como estrategias de preparación de muestras. Las muestras analíticas se analizaron mediante el método desarrollado en el Capítulo 1, utilizando el método automatizado en línea SPE–LC–MS/MS para la cuantificación de la vitamina D y sus principales metabolitos con interés clínico.

Los dos procedimientos de preparación de muestras, la precipitación de proteínas y la SPE automatizada, se compararon en términos de sensibilidad, rango de calibración lineal y efectos de matriz para establecer cuándo se debe usar cada uno. Los rangos lineales de calibración obtenidos tras la precipitación de

proteínas, tanto en plasma como en suero, fueron más cortos que los proporcionados después de la SPE. De acuerdo con los LOQ obtenidos, no se puede recomendar la precipitación de proteínas para la preparación de muestra previa al análisis de los dihidroximetabolitos, ya que estos se encuentran al nivel de pmol/L. Sin embargo, si el objetivo es la determinación de vitamina D y los monohidroximetabolitos, se puede usar la desproteinización. Las diferencias en la sensibilidad que se obtiene tras aplicar cada uno de los protocolos pueden deberse al volumen de muestra analítica inyectada en la columna después de aplicar la precipitación de proteínas: 41.6 µL (considerando todos los pasos involucrados en este procedimiento de preparación de la muestra) frente a 200 µL en el método basado en SPE: 4.8 veces mayor en este último caso.

Se optimizaron las principales variables involucradas en el procedimiento automatizado de SPE para cada tipo de muestra. Las diferentes composiciones de suero y plasma proporcionaron diferencias en las etapas involucradas en la SPE, especialmente en la etapa de carga, en el lavado del cartucho de sorbente y, finalmente, en el tiempo requerido para la elución. La eficiencia de la etapa SPE para cada matriz se evaluó mediante el cálculo del factor de recuperación, que fue próximo al 100% para los dihidroximetabolitos en suero o plasma. Se observó una ligera disminución en el factor de recuperación para los monohidroximetabolitos, que fue más significativo para el 25(OH)D_2 cuando se determinó en plasma (83.0%). La disminución del factor de recuperación fue más significativa para las vitaminas D_2 y D_3, ya que este parámetro estuvo en el entorno del 60% en suero o plasma. Se estudiaron la precisión y los potenciales efectos de la matriz. Los resultados fueron superiores al 89% para todos los analitos en el caso del suero, excepto para la vitamina D_2 (83.3%); para el plasma la precisión fue superior al 90,1%. En general, la precisión calculada para la mayoría de los analitos fue ligeramente mejor (lo que significa que los resultados fueron más cercanos al 100%) en plasma que en suero, a excepción de la vitamina D_3. Sin embargo, una composición más compleja de la muestra de plasma dio lugar a una contribución

del ruido de fondo más alta que la observada para el suero, lo que fue problemático para la cuantificación de niveles bajos de vitamina D y de dihidroximetabolitos.

La influencia del tubo utilizado para la extracción de sangre en la determinación cuantitativa de la vitamina D y sus metabolitos también se evaluó mediante un análisis comparativo de las muestras de suero y plasma recogidas en tubos simples o con gel. En el caso del suero no se encontraron diferencias significativas, lo que significa que el suero recogido en tubos simples y con gel podrían utilizarse indistintamente para el análisis cuantitativo de la vitamina D y sus metabolitos. Tampoco el uso de tubos simples para plasma y tubos con "gel para plasma" resultó crítico para la determinación cuantitativa de los metabolitos de la vitamina D, que son el objetivo principal desde una perspectiva clínica.

Los niveles de vitamina D_3 y de los metabolitos en las muestras de plasma y suero de la cohorte en estudio, también se compararon estadísticamente mediante un test de la t por parejas (nivel de confianza del 95%), mediante el que no se detectaron diferencias significativas entre los niveles de vitamina D_3, $25OHD_3$ y $24,25(OH)_2D_3$ en suero y plasma, pero sí una diferencia significativa en la concentración de $1,25(OH)_2D_3$. Por lo tanto, el plasma parece ser más adecuado que el suero para el análisis cuantitativo de $1,25(OH)_2D_3$, que es relevante para determinar los niveles bajos de la forma activa. Se realizó un análisis de regresión de Passing-Bablok con la concentración obtenida en suero y plasma de la cohorte en estudio. Los coeficientes de regresión oscilaron entre 0.518 para $1,25(OH)_2D_3$ y 0.768 para $25(OH)D_3$. La validez de los modelos lineales se estudió mediante el test de Cusum, que no dio lugar a una desviación significativa de la linealidad para la vitamina D_3 y sus tres metabolitos (valor de *p* de 0.39 para $24,25(OH)_2D_3$ y próximos a 0.86 para el resto del analitos).

Con respecto al estudio sobre la estabilidad de la vitamina D y sus principales metabolitos en suero humano, se probaron durante dos meses cinco estrategias para la conservación de la muestra (temperatura ambiente, refrigeración a 4 °C,

congelado a –20 °C o a –80 °C y liofilización). Además, se evaluó la influencia de los ciclos de congelación/descongelación en los analitos objetivo para establecer el número de ciclos de congelación/descongelación que se pueden realizar sin degradación significativa.

El tiempo de almacenamiento jugó un papel relevante en la estabilidad de la vitamina D y sus metabolitos principales, pero con claras diferencias según el compuesto en cuestión. La forma activa, $25(OH)D_3$, no experimentó cambios significativos de concentración cuando se sometió a cada una de las estrategias de almacenamiento: fue estable durante dos meses independientemente de la temperatura. Por el contrario, la temperatura ambiente produjo una degradación significativa en la vitamina D_3 después de 5 días; en $1,25(OH)_2D_3$ después de 3 días y en $24,25(OH)_2D_3$ desde el primer día. La refrigeración a 4 °C también ejerció diferentes efectos en cada analito: la vitamina D_3 mostró una degradación significativa después de 10 días, el metabolito $24,25(OH)_2D_3$ después de 30 días y el $1,25(OH)_2D_3$ después de dos meses. Finalmente, en condiciones de congelación, los metabolitos mostraron comportamientos singulares: la vitamina D_3 experimentó una degradación significativa después de 10 días de refrigeración, mientras que el metabolito $1,25(OH)_2D_3$ y el $24,25(OH)_2D_3$ se mantuvieron estables durante los dos meses del estudio.

En lo que se refiere a los ciclos de congelación/descongelación de alícuotas de suero almacenadas en el congelador a dos temperaturas diferentes (–20 y -80 °C), se observó un comportamiento diferente para cada analito: la vitamina D_3 y el metabolito $25(OH)D_3$ no sufren alteraciones significativas después de cinco ciclos de congelación/descongelación a cualquiera de las dos temperaturas, lo que puede explicarse por los niveles a los que se encuentran en suero humano (del orden de los nmol/L). La concentración de $24,25(OH)_2D_3$ y $1,25(OH)_2D_3$ disminuyó significativamente después del tercer ciclo de descongelación en las alícuotas almacenadas a las dos temperaturas, debido a los cambios en la composición de la matriz después de cada ciclo de descongelación, causado por la formación de

agregados de varios componentes del suero, principalmente por las proteínas, que pueden interferir en la aplicación de los métodos analíticos.

La liofilización de la muestra se estudió como una alternativa para la conservación de la muestra, ya que este procedimiento estabiliza las muestras biológicas debido a la eliminación del agua, que minimiza la actividad enzimática. Una vez liofilizada la muestra se puede almacenar a temperatura ambiente. Los resultados de este estudio mostraron claramente que los metabolitos $25(OH)D_3$, $1,25(OH)_2D_3$ y $24,25(OH)_2D_3$ no experimentaron cambios significativos después de uno o dos meses a temperatura ambiente. Por el contrario, la vitamina D_3 fue menos estable que sus metabolitos y sufrió una disminución significativa de la concentración después de dos meses. A corto plazo (dos meses), la eliminación del contenido de agua parece desempeñar un papel fundamental para garantizar la estabilidad de los metabolitos de la vitamina D_3 a temperatura ambiente, considerando que la determinación de estos compuestos en alícuotas sin congelar sufrió variaciones significativas.

Sección III: Aplicación de los métodos desarrollados a la determinación masiva de vitamina D y sus metabolitos

Esta sección se centra en la aplicación de las plataformas desarrolladas en diferentes investigaciones clínicas, para la determinación de la cantidad de vitamina D y sus principales metabolitos en suero humano, que fue la muestra recolectada en todos los casos.

Hoy en día, la vitamina D es el caballo de batalla de muchas teorías sobre su comportamiento en diferentes trastornos crónicos de los seres humanos, como diabetes, esclerosis múltiple, enfermedades cardiovasculares, asma, varios tipos de cáncer (de pulmón, colorrectal, de mama o de próstata), enfermedad de Parkinson o fibrosis quística. Por este motivo, la investigación se centra en establecer los

niveles de vitamina D y sus metabolitos principales como un nuevo biomarcador de diferentes patologías.

En esta sección se recoge la aplicación de los métodos desarrollados para el análisis de un total de 2890 muestras de suero humano. Los estudios se centraron en determinar los niveles de los analitos diana en sujetos con esclerosis múltiple, cáncer de mama o en mujeres premenopáusicas (Capítulo 6).

Hay algunos temas pendientes relacionados con el metabolismo de la vitamina D_3, el principal de los cuales es la controversia sobre los valores de los niveles fisiológicos y patológicos de los metabolitos de la vitamina D (abordados en el Capítulo 5). Por lo tanto, el mejor punto de partida para aumentar el conocimiento y apoyar la alta participación de esta vitamina liposoluble en las funciones fisiológicas fue analizar el suero de una cohorte de 558 mujeres y evaluar la variabilidad en la concentración de vitamina D_3 y sus principales metabolitos.

La investigación tuvo como objetivo estimar el metabolismo de la vitamina D_3 en una cohorte de 558 mujeres mediante la evaluación de la incidencia de diferentes factores —la edad, las características antropométricas como el índice de masa corporal (IMC) y el índice cintura–cadera (ICC) y factores estacionales y de localización— en la concentración de vitamina D_3 y metabolitos.

Los resultados de la determinación de los analitos objetivo en la población en estudio fueron: (a) para el calcidiol, una concentración promedio de 47.3 nmol/L (4.8–79.8 nmol/L). Un resultado de acuerdo con los niveles séricos informados previamente para este metabolito en individuos sanos (8–165 nmol/L). (b) Para el colecalciferol, cuya concentración promedio fue de 7.3 nmol/L (1.7–12.2 nmol/L), resultó ser más baja que la proporcionada por Poongkody *et al.*, quienes obtuvieron un rango para individuos normales entre 18 y 150 nmol/L. (c) Para la forma activa, $1,25(OH)_2D_3$, la concentración promedio fue 113.1 pmol/L (43.2–148.5 pmol/L), bastante similar al rango proporcionado por Hollis *et al.* (48-168

pmol/L). (d) Finalmente, la concentración promedio de $24,25(OH)_2D_3$ fue de 3.9 nmol/L (0.3–8.5 nmol/L), ligeramente inferior a los datos informados previamente por Beacher *et al.* como el rango normal en sujetos humanos sanos (2.8–30 nmol/L).

También se calcularon las relaciones entre pares de analitos para evaluar la actividad enzimática en las rutas metabólicas de la vitamina D. La relación $25(OH)D_3/D_3$ fue de 7.0±3.2, indicativa de la actividad enzimática de la 25-hidroxilasa en la primera etapa de hidroxilación. La relación de conversión para la producción de los dos dihidroximetabolitos se calculó mediante las relaciones $24,25(OH)_2D_3/25(OH)D_3$ y $1,25(OH)_2D_3/25(OH)D_3$, que fueron de 0.08±0.04 y de 0.0029±0.002, respectivamente.

Los resultados estudiados en la cohorte se analizaron estadísticamente para asociar el metabolismo de la vitamina D_3 con diferentes factores.

No hubo diferencias significativas en la concentración de vitamina D_3 y sus metabolitos en función de la edad. Con respecto al IMC, solo el nivel de vitamina D_3 se alteró significativamente (valor de p 0.0003); mientras que el valor de ICC proporcionó diferencias significativas para la vitamina D_3 (valor de p 0.001) y dos metabolitos, $25(OH)D_3$ (valor de p 0.02) y $24,25(OH)_2D_3$ (valor de p 0.04).

Los resultados proporcionados por la evaluación de la influencia de los parámetros IMC y ICC en los niveles de los analitos diana sugieren que la obesidad está asociada con la deficiencia de vitamina D_3 a través de una disminución del metabolismo. La vitamina D es liposoluble, por lo tanto se almacena principalmente en el tejido adiposo que sirve como una fuente endógena de vitamina D. Por esta razón, los individuos con IMC y ICC altos presentan niveles de vitamina D en sangre más bajos que los individuos con peso normal. Otra razón que explica la asociación de la obesidad y los niveles de vitamina D es la no

expresión de la 25-hidroxilasa adiposa en individuos obesos, una enzima clave para la regulación de la síntesis de vitamina D.

El metabolito $1,25(OH)_2D_3$ no proporcionó diferencias significativas relacionadas con los valores de IMC y ICC. Este hecho podría justificarse porque la producción de este metabolito tiene lugar en diferentes tejidos (hueso, placenta, próstata, queratinocitos, macrófagos, linfocitos T, células dendríticas varias y glándula paratiroidea), donde se expresa la enzima 1α-hidroxilasa.

También se estudió la influencia del período estacional en los niveles de vitamina D_3 y sus metabolitos. Para ello, las muestras se organizaron según el período estacional en el que se extrajeron: otoño, invierno, primavera y verano. El análisis ANOVA reveló que la vitamina D_3 (valor de p=0.003), $25(OH)D_3$ (p=0.0003) y $24,25(OH)_2D_3$, (p=0) proporcionaron la misma tendencia de variabilidad y sus niveles disminuyeron siguiendo la secuencia verano>otoño>invierno>primavera. Este comportamiento corroboró que la estacionalidad es uno de los principales factores que afectan a los niveles séricos de vitamina D_3 y sus metabolitos. Sin embargo, parece existir un efecto memoria que explicaría por qué los niveles de vitamina D en otoño e invierno estaban por encima de las concentraciones encontradas en primavera.

Se definió un factor espacial según el origen geográfico de las muestras considerando cuatro latitudes en España: 43N, 42N, 41N y 40N. El test ANOVA mostró que las concentraciones de $25(OH)D_3$ (p=0.003) y de $24,25(OH)_2D_3$ (p=0.03) variaron significativamente en función de la latitud. Los niveles más altos encontrados para $25(OH)D_3$ en las latitudes 40N y 43N podrían explicarse porque el área que se ajusta a la latitud 40N se encuentra más al sur que el resto de las áreas, y el área correspondiente a la latitud 43N es un área costera. La exposición a la radiación UV es mayor en estas áreas en comparación con otras latitudes. Por otro lado, el metabolito $24,25(OH)_2D_3$ se encontró en concentraciones más altas en

muestras de individuos de la latitud 41N, que es otra zona costera en el noreste de España.

Como ocurrió con los factores antropométricos, no se encontró asociación entre la estación y/o la latitud con los niveles de $1,25(OH)_2D_3$.

Se abordó un estudio adicional para encontrar la influencia de otros factores, a saber. Actividad física en los últimos 5 años (met-h/semana), estado de la menopausia, ingesta de calcio (g día) e ingesta de energía (kcal) en los niveles de vitamina D en la sangre. Después de la evaluación estadística mediante el test ANOVA, solo la ingesta de calcio y energía contribuyó a explicar significativamente los niveles de vitamina D. El metabolito $24,25(OH)_2D_3$ se asoció significativamente con la ingesta de calcio (p=0.02). El aumento de la concentración de este metabolito con el de la ingesta de calcio sugiere una correlación entre los niveles de calcio y la formación de $24,25(OH)_2D_3$, el principal producto del catabolismo de la vitamina D_3. Algunos estudios ya han sugerido que la ingesta de calcio pueden influir en el consumo metabólico de $25(OH)D_3$, y también parece aumentar la vida media del metabolito $25(OH)D_3$. Estos dos efectos puede que afecten a la concentración de metabolitos relacionados. Con respecto a la ingesta de energía, el análisis reveló diferencias significativas de la relación entre la forma circulante y su precursor, 25 $(OH)D_3$/vitamina D_3 (p=0.03). Atendiendo a estos resultados, hay un efecto de activación/inhibición del metabolismo de la vitamina D_3 atribuido a la ingesta de energía.

Respecto a los centros de investigación nacionales e internacionales que han solicitado el análisis del metabolismo de la vitamina D en diferentes estudios clínicos, en todos los casos el objetivo principal de los análisis fue establecer asociaciones entre las concentraciones de vitamina D y diferentes trastornos como esclerosis múltiple, cáncer de mama o en estados fisiológicos como la menopausia.

Se analizaron un total de 314 muestras de suero, en dos lotes, del Ospedale San Raffaele de Milán, Italia, para determinar los niveles de vitamina D y metabolitos en pacientes con síndromes aislados clínicamente y asociar estos niveles con el riesgo de sufrir esclerosis múltiple (EM). Los principales resultados del estudio del primer lote fueron que los niveles altos de $25(OH)D_3$ se asociaron con un bajo riesgo de esclerosis múltiple y 52% de los pacientes reportaron deficiencia de vitamina D al establecer el nivel de corte en 50 nmol/L. Durante el seguimiento (media de edad de los pacientes 7.17 años), 55 pacientes desarrollaron esclerosis múltiple clínicamente definida (CDMS). Entre los pacientes con niveles medios o altos de $25(OH)D_3$ se identificaron dos grupos, de los que el grupo con una producción significativamente menor de metabolitos activos e inactivos (baja actividad CYP27) tenía un riesgo significativamente mayor de EM. Los resultados del segundo lote están siendo tratados por el grupo de investigación de la Dra. Dalla Costa buscando la correlación entre los niveles basales de vitamina D y la actividad de la enfermedad en pacientes con esclerosis múltiple tratados con fingolimod.

También se analizó un lote de 1104 muestras de suero de un estudio de multi casos-control en España. La conclusión principal fue una asociación inversa entre los niveles séricos de $25(OH)D_3$ y el riesgo de cáncer de mama, que fue más pronunciada en los tumores triplemente negativos. Los autores del estudio finalmente concluyeron que son deseables las estrategias clínicas y de salud pública destinadas a mejorar los niveles de vitamina D, teniendo en cuenta la alta proporción de mujeres con una concentración inadecuada de 25(OH)D.

Un estudio final incluyó el análisis de 1472 muestras de suero de mujeres pre-menopáusicas. Los resultados mostraron que la densidad mamográfica (DM) en mujeres con niveles séricos suficientes de 25(OH)D (>75 nmol/L) fue ligeramente más baja que la DM en mujeres con niveles deficientes, siendo este efecto protector más acentuado entre las mujeres nulíparas. Se comprobó el efecto protector de la vitamina D en la DM y la alta prevalencia de insuficiencia de vitamina D en la

población de la cohorte; por lo tanto, se debe proponer aumentar los niveles de vitamina D como una forma eficaz de prevenir el aumento de la MD posmenopáusica, que puede derivar en el cáncer de mama.

The current rules in the University of Córdoba about the model of doctoral Thesis-Book in the format of articles compilation (published or next to publication) make mandatory a section for the a joint discussion of the results.

The research in this Doctoral Thesis, devoted entirely to vitamin D and its main metabolites in humans, can be divided into three sections as a function of the objectives that coincide with the three principal steps of the analytical research: method development, sample preparation and application to real samples.

Thus, Section I is focused on the development of new methods, Chapter 1 and 2, to increase the number of vitamin D metabolites determined automatically in one single analysis eliminating the human error, and improving sensitivity and selectivity using deuterated internal standards, different columns configurations for the analytical separation and mass spectrometry for determination.

The second section deals with a key and poorly studied step in the analytical process to determine vitamin D: sample preparation. In this section two blood samples (serum and plasma) were evaluated to compare two biological matrices to determine vitamin D metabolites; and two sample preparations (protein precipitation and on-line solid phase extraction, SPE) were tested with the same aim (Chapter 3). In addition, a study of sample stability was carried out, shown in Chapter 4, in which the best storage temperature and freeze/thaw cycles for each of the target compounds were established. Finally, a novel room temperature storage based on sample lyophilization was proposed.

In Section III, and as assessment of the results from the other two previous sections, massive analysis of serum samples from a number of patients cohorts were analyzed. The MCC study (Chapter 5) was focused on the determination of vitamin D metabolites in healthy women and on the statistical analysis of the results. The other studies, which ranged from multiple sclerosis in child, breast cancer in woman and osteoporotic woman, have been collected in Chapter 6, in

order to show the applicability of the analytical platform developed in this Thesis. Annex 4 is a publication based on the results obtained by analysis of samples from one of the massive cohorts.

Section I: Development and improvement of analytical platforms for determination of vitamin D and its metabolites

The growing number of diseases in vitamin D and/or its main metabolites seems to play a crucial role in triggering the interest for the development of new analytical methods for the determination of these compounds. The complexity of the analysis of these metabolites is caused by their structural similarity, hydrophobic nature and thermal and UV-light instability. Recent methods for determination of the main metabolites range from immunoassays (ELISA, RIA, CPB) to those based on liquid or gas chromatographic separation and determination by molecular UV-visible absorption, flame ionization or mass spectrometry (MS).

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods are at present considered the "gold standard", but low concentrations of the analytes in biological matrices make mandatory preconcentration and clean-up steps, for which automated SPE constitutes an excellent tool to facilitate and speed up sample preparation. In addition, the system can be on-line coupled to LC equipment, thus improving robustness and speed of analysis.

On the other hand, determination of 3-epi-25(OH)D_3 and 25(OH)D_3 is a complex task, as both metabolites have identical elemental composition and MRM transitions for quantitation, but different structure, thus leading to overestimation of 25(OH)D_3 concentration in most LC–MS/MS methods, which are unable to differentiate the co-eluted epimers. A new two-dimensional chromatographic platform designed to separate both analytes also allows determination other crucial vitamin D metabolites.

The results provided by this research constitute Chapters 1 and 2 and are discussed below.

The first study was aimed at optimizing and validating an automated on-line SPE–LC–MS/MS platform with analytical features for determination of vitamin D and its main metabolites. The method was targeted at vitamins D_2 and D_3, monohydroxy-metabolites and, mainly, at dihydroxymetabolites of the two forms of vitamin D: $1,25(OH)_2D_2$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The inclusion of vitamin D_2 and its metabolites in the analytical methods is of particular interest when this form is administered as supplement, a common practice in USA and Northern Europe.

The optimization of the SPE step was a crucial analytical step taking into account the low levels at which vitamin D metabolites are present in blood. Sample preparation was focused on the extraction of the analytes and clean-up for interferents removal. Two sorbents (C18 and C8) were studied to select the most appropriate cartridge. Although C18 showed good results for vitamin D, due to its higher lipophilic interaction with this analyte, C8 was selected as this sorbent was the best for mono- and dihydroxymetabolites.

The corresponding SIL-IS was used for each analyte, and the proper chromatographic separation allowed its unequivocal determination. Furthermore, the monitored product ions were equal for each metabolite and its corresponding deuterated standard.

The analytical characteristics of the method made this platform very appropriate to determine the metabolites in serum samples, showing good LODs and LOQs. The method provided within-day variability between 1.5 and 11.5% and between-day variability below 13%, both expressed as RSD. The recovery factor was close to 100% for di- and monohydroxymetabolites, and around 60% for vitamins D_3 and D_2, explained by a low extraction efficiency of the C8 sorbent for the two less polar

forms. Finally, an excellent accuracy was obtained for this method: from 89.3 to 93.6%.

Validation was executed by external quality assessment (DEQAS), by which 10 samples were analyzed to determine total 25(OH)D and other 10 for total $1,25(OH)_2D$. In both cases, the results were within the limits imposed by DEQAS.

The following step was a new analytical method also for vitamin D metabolites in human serum including in this case the 3-epi-$25(OH)D_3$ form. The method involved SPE on-line coupled to two-dimensional liquid chromatography (2DLC) separation and MS/MS detection. A C18 column and a pentafluorophenyl (PFP) column were used for separation of the two $25(OH)D_3$ epimers without affecting the sensitivity and resolution for the other main vitamin D metabolites.

A first comparison between the two columns (PFP and C18) was carried out to get a good separation for subsequent determination of all analytes. Using the PFP column, separation between $25(OH)D_2$, $25(OH)D_3$ and 3-epi-$25(OH)D_3$ was achieved, but with a poor sensitivity for the dihydroxymetabolites. On the other hand, using the C18 column the sensitivity was enough good for all the analytes, but separation of the epimer from the other monohydroxymetabolites was impossible. For this reason a 2DLC configuration was developed to combine the good properties of each column in one single platform. First, the dihydroxymetabolites were separated in the C18 column, followed by the separation of monohydroxymetabolites by the PFP column, and, finally the vitamin D analytes were separated by the C18 column. All the process was designed to be controlled automatically in the elution step by the automated SPE, and an excellent chromatogram was thus provided by MS.

The sensitivity provided by the 2DLC platform was quite similar to that reported in Chapter 1, with a quite similar approach but using only one column for separation. For the new analyte, the epimer, the LOD and LOQ were very similar to those of the

other monohydroxymetabolites and allowed its determination in human serum. Similarly, the precision provided by this platform was similar to that in Chapter 1: between-days variability below 11.6%, and within-day variability below 7.2% for all analytes (always expressed as RSD). The precision for 3-epi-25(OH)D_3 was slightly lower than for the 25(OH)D_3 form, for which the precision was not affected by the 2DLC separation.

The recovery factors were around 100% for the dihydroxymetabolites, and above 95.2% for the monohydroxymetabolites, including the 3-epimer. On the other hand, and as in Chapter 1, the recovery factor decreased to 59.1 and 60.2 for vitamin D_2 and D_3, respectively. The accuracy of the method and potential matrix effects ranged from 83.3 to 96.1, and, 95.5% for the C3-epimer; sensitivity values appropriate for its quantitation in human serum.

Finally, external validation encompassed two ways: a monohydroxymetabolites validation by an NIST standard reference material, and the DEQAS external validation as in Chapter 1. With the NIST standard reference material a triplicate analysis for each of the four concentration levels, was carried out, and the percentage of bias by comparison with the reference values ranged from 2.0 to 8.5%, thus emphasizing the accuracy of the proposed approach for analysis of the three monohydroxymetabolites. Five samples from DEQAS were used to externally validate the dihydroxymetabolites according to the limits established by DEQAS.

Section II: Studies on sample preparation, type of sample and its stability for determination of vitamin D and its metabolites

This section focuses on the sample preparation step (Chapter 3) for determination of the main vitamin D metabolites, and on different strategies to store the serum samples in clinical studies, to avoid degradation issues (Chapter 4). In both studies the principal objective was to improve the sensitivity of the method, considering

the low concentration of the target metabolites in human blood (nmol/L or pmol/L levels).

Different biological samples have been previously tested for analysis of vitamin D and its metabolites (*e.g.,* saliva, cerebrospinal fluid or dried blood spots), but serum and plasma persist as the main biological matrices for determination of these compounds because both can be easily obtained and contain the main metabolites at quantifiable levels. Therefore, sample preparation protocols, which depend on both the use of serum or plasma and the analytes to be determined, had been published, but never compared among them. Two strategies for sample preparation of serum and plasma were assayed (protein precipitation and on-line SPE), which were optimized for each biological matrix.

Other critical variable owing to the nature of vitamin D metabolites is their stability in clinical samples. This is a key aspect for a reliable assessment of the quantitative results in epidemiological research. The behavior of the target analytes from blood extraction to their determination has a critical relevance. How long and under what conditions must the samples be stored were key issues to be studied in depth as the stability of vitamin D metabolites in blood had been insufficiently studied so far. In fact, despite the instability in blood associated to vitamin D metabolites, only that of the 25(OH)D_3 metabolite had been studied.

The results obtained in the study of these issues are discussed in detail below.

The research on the influence of the sample obtained from blood (serum or plasma) for quantitative analysis of the target analytes involved: (i) the type of sample, serum or plasma; (ii) the presence of gel in the tubes for blood collection (used to favor separation of serum or plasma from blood cells); and (iii) the evaluation of protein precipitation or automated SPE as sample preparation strategies. The analytical samples were analyzed by the method developed in

Chapter 1, using the on-line automated SPE–LC–MS/MS method for quantitation of vitamin D and its main metabolites with clinical interest.

The two sample preparation procedures, protein precipitation and automated SPE, were compared in terms of sensitivity, linear calibration range and matrix effects to establish when each should be used. The calibration linear ranges obtained after protein precipitation both in plasma and serum were shorter than those provided after SPE. According to the obtained LOQs, protein precipitation could not be recommended for sample preparation prior to analyze dihydroxymetabolites since they are found at the pmol/L level. Nevertheless, if the target are the monohydroximetabolites vitamin D metabolites, deproteination can be used. The differences in sensitivity provided after using the two preparation methods can be due to the volume of analytical sample injected on-column after applying protein precipitation: 41.6 μL (considering all the steps involved in this sample preparation procedure) *versus* 200 μL for the SPE-based method: 4.8 times higher in the latter case.

The main variables involved in the automated SPE procedure for each type of sample were optimized. The different composition of serum and plasma provided differences in the steps involved in the SPE, especially in the loading step, in washing of the sorbent cartridge and, finally, in the time required for the elution step. The efficiency of the SPE step for each blood matrix was assessed by calculation of the recovery factor. It was around 100% for the dihydroxymetabolites either in serum or plasma. A slight decrease in the recovery factor was observed for the monohydroxymetabolites, which was more significant for 25(OH)D_2 when determined in plasma (83.0%). The decrease of the recovery factor was more significant for vitamins D_2 and D_3, as this parameter was around 60% in serum or plasma. The accuracy and potential matrix effects were studied; the results were above 89% for all analytes in the case of serum, except for vitamin D_2 (83.3%). For plasma, the accuracy of the results was above 90.1%. In general, the accuracy calculated for most analytes was slightly better (which means that the

results were closer to 100%) in plasma than in serum, except for vitamin D_3. Nevertheless, a more complex composition of the plasma sample gave place to a background contribution higher than that observed for serum, which was problematic for quantitation of low levels of vitamin D and dihydroxymetabolites.

The influence of the tube for blood collection on the quantitative determination of vitamin D and its metabolites was also evaluated by comparative analysis of serum and plasma samples collected in either bare or gel-tubes. In the case of serum, no statistical differences were found, which means that serum collected in bare and gel tubes could be indistinctly used for quantitative analysis of vitamin D and its metabolites. Therefore, the use of plasma and plasma-gel tubes is not critical for quantitative determination of vitamin D metabolites, which are the main objective from a clinical perspective.

The levels of vitamin D_3 and metabolites in plasma and serum samples from the cohort under study were also statistically compared by the paired *t*-test (95% confidence level), which allowed detecting no statistical differences between levels of vitamin D_3, $25OHD_3$ and $24,25(OH)_2D_3$ metabolites in serum and plasma, but a significant difference in the concentration of $1,25(OH)_2D_3$. Therefore, plasma seems to be more suited than serum for quantitative analysis of $1,25(OH)_2D_3$, which is relevant to determine low levels of the active form. A Passing-Bablok regression analysis was carried out with the concentration obtained in serum and plasma of the cohort under study. The regression coefficients ranged from 0.518 for $1,25(OH)_2D_3$ to 0.768 for $25(OH)D_3$. The validity of the linear models was studied by the Cusum test, which reported no significant deviation from linearity for vitamin D_3 and its three metabolites (*p*-value of 0.39 for $24,25(OH)_2D_3$ and close to 0.86 for the rest of the analytes).

Concerning the study on stability of vitamin D and its principal metabolites in human serum, five strategies for sample storage (room temperature, refrigeration at 4 °C, frozen at –20 °C or at –80 °C, and lyophilization) were tested for two

months. Furthermore, the influence of freeze/thaw cycles on the target analytes was also evaluated to establish the number of freeze/thaw cycles that can be used without significant degradation.

The storage time played a relevant role in the stability of vitamin D and its principal metabolites, but with clear differences depending on the target compound. The active form, 25(OH)D_3, did not experience significant concentration changes when subjected to any of the storage strategies: it was stable for two months independently of the storage temperature. On the contrary, room temperature causes significant degradation in vitamin D_3 after 5 days, in 1,25$(OH)_2D_3$ after 3 days, and in 24,25$(OH)_2D_3$ since the first day. Refrigeration at 4 °C also exerted different effect on each analyte: vitamin D_3 showed significant degradation after 10 days, 24,25$(OH)_2D_3$ after 30 days, and 1,25$(OH)_2D_3$ after two months. Finally, under freezer conditions the metabolites showed singular behaviors: vitamin D_3 experienced significant degradation after refrigerated for 10 days; 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ were stable during the two-month study.

In dealing with freeze/thaw cycles of serum aliquots of the pool stored in the freezer at two different temperatures (–20° C and –80 °C), different behavior was observed for each analyte: vitamin D_3 and 25(OH)D_3 did not suffer significant alterations after five freeze/thaw cycles at any of the two temperatures, which can be explained by their levels found in human serum (at the nmol/L). The concentration of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ significantly decreased after the third thaw cycle in aliquots stored at the two temperatures, due to changes in matrix composition after each defrosting cycle caused by formation of aggregates of various serum components such as major proteins, which may interfere with the analytical methods.

Sample lyophilization was studied as an alternative for sample storage since this procedure stabilizes biological samples due to water removal, which minimizes enzymatic activity. Once the sample is lyophilized, it can be stored at room

temperature. The results of this study clearly showed that $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, did not experience significant changes after one or two months at room temperature. Contrarily, vitamin D_3 was less stable than its metabolites and suffers a significant decay in concentration after two months. At short term (two months) the removal of water content seems to play a critical role to guarantee the stability of vitamin D_3 metabolites at room temperature, considering that the determination of these compounds in unfrozen aliquots suffered from significant variations.

Section III: Application of the developed methods to the massive determination of vitamin D and its metabolites

This section is focused on the application of the developed platforms to different clinical investigations, supported on the determination of the amount of vitamin D and its principal metabolites in human serum, which was the sample collected in all cases.

Nowadays, vitamin D is the battle horse of many theories about its behavior under different chronic disorders of humans, such as diabetes, multiple sclerosis, cardiovascular diseases, asthma, several types of cancer —lung, colorectal, breast or prostate cancers—, Parkinson disease or cystic fibrosis. For this reason the research is focused on establishing the levels of vitamin D and its principal metabolites as a novel biomarker of different pathologies.

In this section, a total of 2890 human serum samples were analyzed by the developed methods. The studies were focused on determining the levels of the target analytes in subjects with multiple sclerosis, breast cancer or in pre-menopausal women (Chapter 6).

There are some pending issues related to vitamin D_3 metabolism, the main of which is the controversy on values of physiological and pathological levels of vitamin D metabolites (approached in Chapter 5). Therefore, the best starting

point to increase the knowledge and support the high involvement of this liposoluble vitamin in physiological functions was to analyze the serum from a cohort of 558 women, and evaluate the variability in the concentration of vitamin D_3 and its main metabolites.

The research was aimed at estimating the metabolism of vitamin D_3 in a cohort of 558 control women by evaluating the incidence of different factors —*viz.,* age, anthropometric features such as the body mass index (BMI) and waist-to-hip ratio (WHR), and seasonal and location factors— on the concentration of vitamin D_3 and metabolites.

The results from the analysis the taget analytes in the population under study were: (a) for calcidiol, an average concentration of 47.3 nmol/L (4.8–79.8 nmol/L). A result in agreement with previously reported serum levels for this metabolite in healthy individuals (8–165 nmol/L). (b) For cholecalciferol, whose average concentration was 7.3 nmol/L (1.7–12.2 nmol/L), lower than that provided by Poongkody *et al.*, who reported a range for normal individuals between 18 and 150 nmol/L. (c) For the active form, $1,25(OH)_2D_3$, the average concentration was 113.1 pmol/L (43.2–148.5 pmol/L), quite similar to the range provided by Hollis *et al.* (48–168 pmol/L). (d) Finally, the average concentration of $24,25(OH)D_3$ was 3.9 nmol/L (0.3–8.5 nmol/L), slightly lower than the data previously reported by Beacher *et al.* as the normal range in healthy human subjects (2.8–30 nmol/L).

Also the ratio values between pairs of analytes were calculated to evaluate the enzymatic activity in the vitamin D pathways. Thus, the ratio $25(OH)D_3/D_3$ was 7.0±3.2, indicative of the enzymatic activity of 25-hydroxylase in the first hydroxylation step. The conversion rate for production of the two dihydroxymetabolites was calculated by the $24,25(OH)_2D_3/25(OH)D_3$ and $1,25(OH)_2D_3/25(OH)D_3$ ratios, which were 0.08±0.04 and 0.0029±0.002, respectively.

The results studied in the cohort were analyzed statistically to associate vitamin D_3 metabolism with different factors.

There were not significant differences in the concentration of vitamin D_3 and its metabolites as a function of age. Concerning the BMI, only the level of vitamin D_3 was significantly altered (*p*-value 0.0003); while the value of WHR reported significant differences for vitamin D_3 (*p*-value 0.001) and two metabolites, namely $25(OH)D_3$ (*p*-value 0.02) and $24,25(OH)_2D_3$ (*p*-value 0.04).

The results provided by evaluation of the influence of BMI and WHR parameters on the levels of the target analytes suggest that obesity is associated with vitamin D_3 deficiency through a decrease of metabolism. Vitamin D is liposoluble, therefore, it is mainly stored in the adipose tissue that serves as an endogenous source of vitamin D. For this reason, individuals with high BMI and WHI present levels of vitamin D in blood lower than normal weight individuals. One other reason explaining the association of obesity and vitamin D levels is the suppressed expression of adipose 25-hydroxylase in obese individuals, a key enzyme for regulation of vitamin D synthesis.

The $1,25(OH)_2D_3$ metabolite did not report significant differences related to the values of BMI and WHR. This fact could be justified because the production of this metabolite takes place in different tissues (bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells, several and the parathyroid gland), where the enzyme 1α-hydroxylase is expressed.

The influence of the seasonal period on the levels of vitamin D_3 and its metabolites was also studied. For this purpose, the samples were organized according to the seasonal period in which they were extracted: fall, winter, spring and summer. The ANOVA test revealed that vitamin D_3 (*p*-value 0.003), $25(OH)D_3$ (*p*-value 0.0003) and $24,25(OH)_2D_3$, (*p*-value 0) provided the same variability trend, and their levels decreased by following the sequence summer>fall>winter>spring. This

behavior corroborated that the season is one of the main factors that affects the serum levels of vitamin D_3 and its metabolites. However, a memory effect seems to exist that would explain why vitamin D levels in fall and winter were above the concentrations found in spring.

A spatial factor was defined according to the geographical origin of samples by considering four latitudes in Spain: 43N, 42N, 41N and 40N. The ANOVA test pointed out that the concentrations of $25(OH)D_3$ (*p*-value 0.003) and $24,25(OH)_2D_3$ (*p*-value 0.03) varied significantly as a function of the latitude. Higher levels found for $25(OH)D_3$ in 40N and 43N latitudes could be explained by the area that fits 40N latitude is located further South than the rest of the areas, and the area pertaining to 43N latitude is a coastal area. Exposure to UV irradiation should be higher in these areas as compared to other latitudes. On the other hand, $24,25(OH)_2D_3$ was found at higher concentrations in samples from individuals from 41N latitude, which is another coastal area in the northeast of Spain.

As occurred with anthropometric factors, no association was found between season and/or latitude with levels of $1,25(OH)_2D_3$.

An additional study was addressed at finding the influence of other factors —*viz*. physical activity in the last 5 years (met-h/week), menopause status, calcium intake (g/day) and energy intake (kcal)— on vitamin D levels in blood. After statistical evaluation by ANOVA test, only calcium and energy intake contributed to explain significantly the levels of vitamin D. The $24,25(OH)_2D_3$ metabolite was significantly associated with calcium intake (*p*-value 0.02). The increase of the concentration of this metabolite with the increased intake of calcium suggests a correlation between calcium levels and formation of $24,25(OH)_2D_3$, the main product of vitamin D_3 catabolism. Some studies have already suggested that calcium intake could influence $25(OH)D_3$ metabolic consumption, and also seems to increase the half-life of $25(OH)D_3$. These two effects should affect the concentration of related metabolites. Concerning the energy intake, the analysis

revealed significant differences of the ratio between the circulating form and its precursor, 25(OH)D_3/vitamin D_3 (*p*-value 0.03). Attending to these results, there is an activation/inhibition effect of the vitamin D_3 metabolism ascribed to the energy intake.

Concerning the national and international research centers that have demanded for the analysis of vitamin D metabolism in different clinical studies, in all cases the main aim of the analyses was to establish associations between vitamin D concentrations and different disorders as multiple sclerosis, breast cancer, or physiological states such as menopause.

A total of 314 serum samples, in two batches from the Ospedale San Raffaele in Milan, Italy, were analyzed to determine the levels of vitamin D and metabolites in patients with clinically isolated syndromes and associate these levels with the risk for suffering multiple sclerosis (MS). The main results of the study of the first batch was that high levels of 25(OH)D_3 were associated with a low risk from multiple sclerosis. A total of 52% of patients reported vitamin D deficiency by setting the cut-off level at 50 nmol/L. During follow-up (median: 7.17 years), 55 patients developed clinically definite multiple sclerosis (CDMS). Among patients with medium or high levels of 25(OH)D_3, two clusters were identified, where the cluster with a significant lower production of active and inactive metabolites (low CYP27 activity) had a significantly higher risk of MS. The results of the second batch are being treated by the research group of Dr. Dalla Costa searching for correlation between basal vitamin D levels and disease activity in multiple sclerosis patients treated with fingolimod.

A batch of 1104 serum samples from a multicase-control study in Spain were also analyzed. The principal conclusion was an inverse association between 25(OH)D_3 serum levels and breast cancer risk, which was more pronounced in triple negative tumors. The authors of the study finally concluded that public health and clinical

strategies aimed at improving vitamin D levels would be desirable, taking into account the high proportion of women with inadequate concentration of 25(OH)D.

A final study involved the analysis of 1472 serum samples from premenopausal women. The results showed that the mammographic density (MD) in women with sufficient serum levels of 25(OH)D (>75 nmol/L) was slightly lower than the MD of women with deficient levels, being this protective effect more accentuated among nulliparous women. The protective effect of vitamin D on MD and the high prevalence of vitamin D insufficiency in the cohort population were proved; therefore, to enhance vitamin D levels should be proposed as an effective way to prevent postmenopausal increased MD, which can derive in breast cancer.

CONCLUSIONES

CONCLUSIONS

Las conclusiones más destacadas de la investigación dedicada al desarrollo de nuevas plataformas para la determinación de la vitamina D y sus principales metabolitos se pueden resumir como sigue::

- El método automatizado para el análisis cuantitativo de la vitamina D y sus principales metabolitos proporciona una rápida separación cromatográfica de las vitaminas D_3 y D_2 y los metabolitos $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$, $25(OH)D_3$, $25(OH)D_2$ en muestras de suero, utilizando un volumen de muestra pequeño (250 µL). El método se mejoró utilizando el deuterado correspondiente a cada analito para compensar los efectos de supresión de la ionización y otros errores producidos durante el análisis.

- Se ha desarrollado otra plataforma, basada en la separación cromatográfica bidimensional, para agregar la separación de los epímeros de $25(OH)D_3$ al resto de los metabolitos de la vitamina D. Por lo tanto, se determinan ocho analitos en un solo análisis con suficiente sensibilidad para su cuantificación en suero humano (a nivel de nmol/L o pmol/L).

- Ambas plataformas se han caracterizado y validado mediante programas externos utilizando muestras proporcionadas por DEQAS, para la determinación del total de $25(OH)D$ y $1,25(OH)_2D$, y un material de referencia certificado por el NIST para validar la determinación individual de los epímeros del $25(OH)D_3$.

Los estudios dirigidos a conocer el efecto de la preparación de la muestra en los metabolitos de la vitamina D, utilizando suero o plasma humanos, dieron como resultado las siguientes conclusiones:

- La preparación de la muestra basada en SPE proporciona , para todos los metabolitos de la vitamina D, límites de cuantificación más bajos que la precipitación de proteínas debido a la diferente cantidad de muestra inyectada en la columna: 4.8 veces mayor en el caso de la SPE. Este comportamiento hace que la etapa de precipitación de proteínas sea inadecuada para la preparación de muestras cuando los analitos diana son los dihidroximetabolitos.

- El tipo de matriz biológica (suero o plasma) influye en la sensibilidad del método, de forma que el plasma es mejor para determinar los dihidroximetabolitos, mientras que el suero es el más adecuado para la determinación de los dos monohidroximetabolitos.

- El uso de tubos de recolección para suero o plasma con o sin recubrimiento de gel demostró no ser estadísticamente significativo para todos los analitos diana, excepto para la vitamina D_3 en plasma, que proporcionó una mayor concentración cuando la muestra se recogió en tubos de plasma.

- Las recomendaciones establecidas en la Tesis en lo que se refiere a la conservación de la muestra (referidas tanto al tiempo de almacenamiento como a los ciclos de congela-ción/descongelación) deben mantenerse para la medición de todos los metabolitos de la vitamina D_3 en estudios clínicos, donde la sensibilidad es un parámetro crítico, concretamente:

  (1) Los metabolitos de la vitamina D_3 son estables durante dos meses cuando las muestras de suero se conservaron en un congelador a –20 o –80 °C.

(2) El número de ciclos de congelación/descongelación debe limitarse a dos, si los analitos son los dos metabolitos dihidroxilados; mientras que el $25(OH)D_3$ y la vitamina D_3 se pueden determinar después de cinco ciclos sin ningún cambio significativo.

(3) La liofilización y posterior almacenamiento a temperatura ambiente son una estrategia fácil, rápida y robusta para conservar las muestras que se utilizarán en los estudios clínicos.

La aplicación masiva de las plataformas desarrolladas para la determinación de los analitos diana en muestras para de investigaciones clínicas demostró su funcionalidad a través de los siguientes casos:

- Un estudio completo del metabolismo de la vitamina D_3 en una cohorte de 558 mujeres.
- Los niveles de $25(OH)D_3$ y $1,25(OH)_2D_3$ en suero de la cohorte estudiada coincidieron con los informados previamente en individuos normales. Los niveles para $24,25(OH)_2D_3$ y vitamina D_3, estuvieron por debajo de los niveles normales.
- La relación entre los analitos podría usarse como indicativo de la actividad enzimática, mostrando un comportamiento global de la ruta enzimática involucrada en el metabolismo de la vitamina D.
- El análisis estadístico de los datos ha puesto de manifiesto que existen varias variables que afectan a los niveles de vitamina D: los valores altos de IMC o ICC están directamente correlacionados con niveles de vitamina D inferiores a los normales, mientra la obesidad parece estar asociada con la deficiencia de vitamina D. Los aspectos estacionales y geográficos influyen en los niveles de vitamina D de la cohorte estudiada, como un claro efecto de la radiación solar UV.

- Tanto la ingesta de calcio como de calorías pueden afectar el metabolismo de la vitamina D y su control tendría un impacto en los niveles de vitamina endógena.
- Tres centros de investigación nacionales e internacionales han solicitado el análisis de la vitamina D y sus metabolitos en 2890 muestras de suero para determinar el metabolismo de la vitamina D en diferentes estudios clínicos realizados a lo largo del desarrollo de la Tesis.
- En todos los casos, el objetivo principal de los análisis fue establecer asociaciones entre las concentraciones de vitamina D y diferentes trastornos como esclerosis múltiple, cáncer de mama o estados fisiológicos como la menopausia.

The most outstanding conclusions from the research devoted to developing new platforms to determine vitamin D and its principal metabolites can be summarized as follows:

- The automated method developed for quantitative analysis of vitamin D and its main metabolites provides a fast chromatographic separation of vitamins D_3 and D_2 and the $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$, $25(OH)D_3$, $25(OH)D_2$ metabolites in serum samples, using a small sample volume (250 µL) The platform was improved using SIL-ISs to compensate for ionization suppression effects and other errors produced along the analysis.

- Another platform, based on two-dimensional chromatographic separation, has been developed to add the separation of the epimers of $25(OH)D_3$ to the rest of vitamin D metabolites. Thus, eight analytes are determined in one single analysis with enough sensitivity for their quantitation in human serum (at the nmol/L or pmol/L level).

- Both platforms have been characterized and validated by external programs using samples provided by DEQAS —for determination of total 25(OH)D and $1,25(OH)_2D$—, and a standard reference material certified by NIST to validate the individual determination of the $25(OH)D_3$ epimers.

The studies aimed at knowing the effect of sample preparation on vitamin D metabolites, either from human serum or plasma, yielded the following conclusions:

- Sample preparation based on SPE provides lower quantitation limits for all vitamin D metabolites than protein precipitation due to the different amount of sample injected on-column: 4.8 times

higher in the case of SPE. This behavior makes the protein precipitation step inappropriate for sample preparation when dihydroxymetabolites are the target analytes.

- The type of biological matrix (serum or plasma) influences the sensitivity of the method in such as way that plasma is better to determine the dihydroxymetabolites, while serum is the best for determination of the two monohydroxymetabolites.

- The use of collection tubes for serum or plasma with or without gel coating showed not be statistically significant for all target analytes, except for vitamin D_3 in plasma, which yielded higher concentration when the sample was collected in plasma tubes.

- The recommendations established in the Thesis referred to storage (both time and freeze/thaw cycles) should be kept for the measurement of all vitamin D_3 metabolites in clinical studies, where the sensitivity is a critical parameter, namely:

  (1) Vitamin D_3 metabolites are stable for two months when serum samples were stored in a freezer at –20 °C or –80 °C.

  (2) The number of freeze/thaw cycles should be limited to two, if the target analytes are the two dihydroxy metabolites; whereas $25(OH)D_3$ and vitamin D_3 would be accurately determined after five cycles without any significant effect.

  (3) Lyophilization and room temperature storage is an easy, fast and robust strategy to preserve temperature samples to be used in clinical studies.

The massive application of the developed platforms to the determination of the target analytes in samples from clinical investigaciones proved their functionality through the following cases:

- A complete study of the metabolism of vitamin D_3 in a 558-women cohort.
- The levels of $25(OH)D_3$, and $1,25(OH)_2D_3$ in serum from the studied cohort were in agreement with those previously reported in normal individuals. The levels for $24,25(OH)_2D_3$ and vitamin D, were below the reported normal levels.
- The ratio between given target analytes could be used as indicative of the enzymatic activity, showing a global behavior of the enzymatic pathway involved in the metabolism of vitamin D.
- The statistical analysis of the data shows several variables that affect the levels of vitamin D: high values of BMI or WHR are directly correlated with lower than normal levels of vitamin D, and obesity seems to be associated with vitamin D deficiency. Seasonal and geographical aspects influence the levels of vitamin D of the studied cohort, as a clear effect of the UV sun irradiation.
- Both calcium and energy intake can affect vitamin D metabolism and a control of them would have an impact on the endogenous vitamin levels.
- Three national and international research centers have demanded for the analysis of vitamin D and metabolites in 2890 serum samples to determine vitamin D metabolism in different clinical studies carried out along the development of the Thesis.
- In all cases the main aim of the analyses was to establish associations between vitamin D concentrations and different disorders as multiple sclerosis, breast cancer, or physiological states such as menopause.

ANEXOS

ANNEXES

Anexo I

Annex I

Revisión bibliográfica sobre el papel del sudor como muestra analítica /Review on the role of sweat as analytical sample

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Review

Sweat: A sample with limited present applications and promising future in metabolomics

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ABSTRACT

Sweat is a biofluid with present scant use as clinical sample. This review tries to demonstrate the advantages of sweat over other biofluids such as blood or urine for routine clinical analyses and the potential when related to metabolomics. With this aim, critical discussion of sweat samplers and equipment for analysis of target compounds in this sample is made. Well established routine analyses in sweat as is that to diagnose cystic fibrosis, and the advantages and disadvantages of sweat *versus* urine or blood for doping control have also been discussed. Methods for analytes such as essential metals and xenometals, ethanol and electrolytes in sweat in fact constitute target metabolomics approaches or belong to any metabolomics subdiscipline such as metallomics, ionomics or xenometabolomics. The higher development of biomarkers based on genomics or proteomics as omics older than metabolomics is discussed and also the potential role of metabolomics in systems biology taking into account its emergent implementation. Normalization of the volume of sampled sweat constitutes a present unsolved shortcoming that deserves investigation. Foreseeable trends in this area are outlined.

Abbreviations: ANOVA, analysis of variance; CE, capillary electrophoresis; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Da, Dalton; DAD, diode array detector; DCD, dermcidin; EI, electron impact ionization; EIA, enzyme immunoassay; ELISA, enzyme linked immunoassay; ESI, electrospray ionization; FID, flame ionization detector; GC, gas chromatograph/gas chromatography; GHB, gamma hydroxybutyrate; HMDB, human metabolome data base; ISE, ion selective electrode; LC, liquid chromatograph/liquid chromatography; LLE, liquid–liquid extraction; LOQ, limit of quantitation; METLIN, metabolite and tandem MS data base; MS, mass spectrometer/mass spectrometry; MRMMS, multiple reaction monitoring mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PCA, principal components analysis; PIP, prolactin inducible protein; PLS-DA, partial least squares-discriminant analysis; pROC, partial receiver operating characteristics; R, Robert and Ross programme language; RIA, radio immunoassay; SPE, solid-phase extraction; TOF, time-of-flight; UV, ultraviolet.

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Anexo II

Annex II

Publicaciones derivadas del proyecto MCC/
Publications from the MCC project

ARTICLE IN PRESS

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Serum 25-hydroxyvitamin D and breast cancer risk by pathological subtype (MCC-Spain)

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ABSTRACT

Epidemiologic evidence on the association between vitamin D and breast cancer is still inconclusive. This study analyzes the association between serum 25-hydroxyvitamin D (25(OH)D) and breast cancer risk by pathologic subtype, stage at diagnosis and specific breast cancer risk factors. We conducted a population-based multicase-control study where 546 histologically-confirmed breast cancer cases and 558 population controls, frequently matched by geographic area, age and body mass index, were recruited in 12 Spanish provinces (MCC-Spain). Information was collected by a questionnaire and plasma 25(OH)D was measured by solid-phase extraction on-line coupled to liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS). Odds ratios and 95% confidence intervals were calculated using logistic and multinomial mixed regression models. We found a clear protective effect between 25(OH)D levels and breast cancer risk, with a significant dose-response trend (OR per 10 nmol/L = 0.88; 95%CI = 0.82–0.94). While no differences were observed between pre and postmenopausal women, stage at diagnosis, or across strata of the main breast cancer risk factors, the protection was more pronounced for triple negative tumors (OR per 10 nmol/L = 0.64; p-heterogeneity = 0.038). Similar results

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Serum 25-Hydroxyvitamin D and mammographic density in premenopausal Spanish women

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Abstract

The role of vitamin D in mammographic density (MD) is still unclear (inconsistent). This study examines the association between serum 25-hydroxyvitamin D (25(OH)D) and MD, overall and by specific women characteristics. We conducted a cross-sectional study where 1409 premenopausal women were recruited in a breast radiodiagnosis unit of Madrid City Council. Information was collected by a questionnaire and plasma 25(OH)D was measured by solid-phase extraction on-line coupled to liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS). Percent MD was assessed using a semi-automated computer tool (DM-Scan). Multivariable linear regression models were used to quantify the associations, categorizing 25(OH)D levels (nmol/L) into 3 groups according to the cutoffs established by the US Endocrine Society. Models were adjusted for age, education, body mass index, age at menarche, parity, previous breast biopsies, family history of breast cancer, physical activity, energy intake, use of corticoids, hypercholesterolemia and day of sample extraction. Mean serum 25(OH)D levels was 49.4 $\pm$ 18.9 nmol/L. women with sufficient concentrations of 25(OH)D showed a slight decrease in MD ($\beta_{>75nmol/L}$=-3.31; p=0.042), with a no significant dose-response trend ($\beta_{per\ 10\ nmol/L}$=-0.32; p=0.195). No differences were observed according to women characteristics except for parity, where a stronger protective effect was seen in nulliparous ($\beta_{>75nmol/L}$ =-13.14; p-heterogeneity=0.007). In light of the protective effect of vitamin D on MD and the high prevalence of vitamin D insufficiency in our population, improving these levels could be an effective measure for the prevention of health problems related to the lack of this essential vitamin.

Anexo III

Annex III

Investigación realizada en colaboración con el Departamento de Farmacología, Toxicología y Medicina Legal y Forense de la Facultad de Veterinaria de la UCO/Research developed in collaboration with the Department of Pharmacology, Toxicology and Legal and Forensic Medicine, Faculty of Veterinary, UCO

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Pharmacokinetics and pharmacodynamics of ramipril and ramiprilat after intravenous and oral doses of ramipril in healthy horses

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ABSTRACT

The pharmacokinetics and pharmacodynamics (PK/PD) of the angiotensin-converting enzyme inhibitor (ACEI) ramiprilat after intravenous (IV) and oral (PO) administration of ramipril have not been evaluated in horses. This study was designed to establish PK profiles for ramipril and ramiprilat as well as to determine the effects of ramiprilat on serum angiotensin converting enzyme (ACE) and to select the most appropriate ramipril dose that suppresses ACE activity. Six healthy horses in a cross-over design received IV ramipril 0.050 mg/kg, PO at a dose of 0 (placebo), and 0.050, 0.10, 0.20, 0.40 and 0.80 mg/kg ramipril. Blood pressures were measured and blood samples obtained at different times. Serum ramipril and ramiprilat concentrations and serum ACE activity were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and spectrophotometry, respectively.

Systemic bioavailability of ramiprilat after PO ramipril was 6–9%. Percentages of maximum ACE inhibitions from baseline were 98.88 (IV ramipril), 5.31 (placebo) and 27.68, 39.27, 46.67, 76.13 and 84.27 (the five doses of PO ramipril). Blood pressure did not change during the experiments. Although oral availability of ramiprilat was low, ramipril has sufficient enteral absorption and bioconversion to ramiprilat to induce serum ACE inhibitions of almost 85% after a dose of 0.80 mg/kg ramipril. Additional research on ramipril administration in equine patients is indicated.

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Pharmacokinetic/pharmacodynamic modeling of benazepril and benazeprilat after administration of intravenous and oral doses of benazepril in healthy horses

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ABSTRACT

Pharmacokinetic and pharmacodynamic (PK/PD) properties of the angiotensin-converting enzyme inhibitor (ACEI) benazeprilat have not been evaluated in horses. This study was designed to establish PK profiles for benazepril and benazeprilat after intravenous (IV) and oral (PO) administration of benazepril using a PK/PD model. This study also aims to determine the effects of benazeprilat on serum angiotensin converting enzyme (ACE), selecting the most appropriate dose that suppresses ACE activity. Six healthy horses in a crossover design received IV benazepril at 0.50 mg/kg and PO at doses 0 (placebo), 0.25, 0.50 and 1.00 mg/kg. Blood pressures (BP) were measured and blood samples were obtained at different times in order to measure serum drug concentrations and serum ACE activity, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and spectrophotometry, respectively. Systemic bioavailability of benazeprilat after PO benazepril was 3–4%. Maximum ACE inhibitions from baseline were 99.63% (IV benazepril), 6.77% (placebo) and 78.91%, 85.74% and 89.51% (for the three PO benazepril doses). Significant differences in BP were not found. Although oral availability was low, benazeprilat 1.00 mg/kg, reached sufficient serum concentrations to induce long lasting serum ACE inhibitions (between 88 and 50%) for the first 48 h. Additional research on benazepril administration in equine patients is indicated.

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Anexo IV

Annex IV

Investigación realizada en colaboración con el Departamento de Agronomía de la Facultad de Ciencias de la UCO/Research developed in collaboration with the Department of Agronomy, Faculty of Sciences, UCO.

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Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey

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Hydroxytyrosol (PubChem CID: 82755)
Oleacein (3,4-DHPEA-EDA) (PubChem CID: 18684078)
Oleocanthal (p-HPEA-EDA) (PubChem CID: 16681728)
Oleuropein aglycon (3,4-DHPEA-EA) (PubChem CID: 124202093)
Luteolin (PubChem CID: 5280445)
Apigenin (PubChem CID: 5280443)

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Oleacein
Oleuropein aglycon
Ligstroside aglycon
LC-MS/MS

ABSTRACT

Despite the evident influence of the cultivar on olive oil composition, few studies have been devoted to exploring the variability of phenols in a representative number of monovarietal olive oils. In this study, oil samples from 80 cultivars selected for their impact on worldwide oil production were analyzed to compare their phenolic composition by using a method based on LC–MS/MS. Secoiridoid derivatives were the most concentrated phenols in virgin olive oil, showing high variability that was significantly due to the cultivar. Multivariate analysis allowed discrimination between four groups of cultivars through their phenolic profiles: (i) richer in aglycon isomers of oleuropein and ligstroside; (ii) richer in oleocanthal and oleacein; (iii) richer in flavonoids; and (iv) oils with balanced but reduced phenolic concentrations. Additionally, correlation analysis showed no linkage among aglycon isomers and oleocanthal/oleacein, which can be explained by the enzymatic pathways involved in the metabolism of both oleuropein and ligstroside.

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Anexo V

Annex V

Investigación realizada en colaboración con el Laboratorio de Farmacognosia y Química de los Productos Naturales de la Facultad de Farmacia de la Universidad de Atenas/Research developed in collaboration with the Laboratory of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens.

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Oleocanthalic Acid, a Chemical Marker of Olive Oil Aging and Exposure to a High Storage Temperature with Potential Neuroprotective Activity

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Supporting Information

ABSTRACT: The investigation of olive oils stored for a period of 24 months under appropriate conditions (25 °C, dark place, and airtight container) led to the identification of a new major phenolic ingredient, which was named oleocanthalic acid. The structure of the new compound was elucidated using one- and two-dimensional nuclear magnetic resonance in combination with tandem mass spectrometry. The new compound is an oxidation product of oleocanthal and is found in fresh oils in very low concentrations. The concentration of oleocanthalic acid increased with storage time, while the oleocanthal concentration decreased. A similar increase of the oleocanthalic acid/oleocanthal ratio was achieved after exposure of olive oil to 60 °C for 14 days. Although the presence of an oxidized derivative of decarboxymethylated ligstroside aglycon had been reported, it is the first time that its structure is characterized. The isolated compound could induce the expression of amyloid-β major transport proteins as well as tight junctions expressed at the blood−brain barrier, suggesting that oleocanthalic acid could be beneficial against Alzheimer's disease.

KEYWORDS: *olive oil, phenolics, stability, NMR, LC−MS/MS, oleocanthal, oleocanthalic acid, Alzheimer's disease*

DOI: 10.1021/acs.jafc.8b00561
J. Agric. Food Chem. 2018, 66, 7337−7346

Anexo VI

Annex VI

Investigación realizada en colaboración con otros miembros del grupo al que pertenece el doctorando/Research developed in collaboration with other members of our research group

Talanta 193 (2019) 29–36

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Talanta

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Determination of primary fatty acid amides in different biological fluids by LC–MS/MS in MRM mode with synthetic deuterated standards: Influence of biofluid matrix on sample preparation

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ARTICLE INFO

Keywords:
Primary fatty acid amides
Liquid chromatography–tandem mass spectrometry
Stable isotopically labeled internal standard
Human biofluids
Sample preparation

ABSTRACT

The recent growing interest in primary fatty acid amides (PFAMs) is due to the broad range of physiological effects they exhibit as bioindicator of pathological states. These bioactive lipids are usually in biological samples at the nanomolar level, making their detection and identification a challenging task. A method for quantitative analysis of seven main PFAMs (lauramide, myristamide, linoleamide, palmitamide, oleamide, stearamide and behenamide) in four human biofluids —namely, urine, plasma, saliva and sweat— is here reported. Two sample preparation procedures were compared to test their efficiency in each biofluid: solid-phase extraction (SPE) and protein precipitation. The latter was the best for plasma and urine, while the analysis of saliva and sweat required an SPE step for subsequent suited determination of PFAMs. Detection of the seven metabolites was performed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) in multiple reaction monitoring (MRM) mode. Quantitative analysis was supported on the use of stable isotopically labeled internal standards (SIL-ISs) in the calibration method, which required the synthesis of each IS from the precursor deuterated fatty acids. Detection limits for the target analytes were within 0.3–3 ng mL^{-1}. The method was applied to a small cohort of male and female volunteers (n = 6) to estimate the relative concentration profiles in the different biofluids. The analytical features of the method supported its applicability in clinical studies aimed at elucidating the role of PFAMs metabolism.

Abbreviations: ACN, acetonitrile; c-μSPE, centrifugal micro-solid phase extraction; EIC, extracted ion chromatogram; FAAH, fatty acid amide hydrolase; FA, formic acid; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; MeOH, methanol; MRM, multiple reaction monitoring; PFAMs, primary fatty acid amides; PIM, product ion monitoring; RSD, relative standard deviation; SIL-ISs, stable isotopically labeled internal standards; TIC, total ion chromatogram

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Available online 25 September 2018

Anexo VII

Annex VII

Comunicaciones orales o en carteles en 7 congresos (3 nacionales y 4 internacionales)/Oral and poster communications in 7 meetings (3 national and 4 international meetings).

1. Comunicación Flash

Contributions to the metabolomics of vitamin D for research of disease biomarkers.

III Scientific Congress of Training Researchers in Agribusiness by CeiA3 (2014).

2. Comunicación Flash

Contributions to the analysis of vitamin D for the study of metabolic diseases.

VI Young Reseachers Meeting of the Maimónides Institute of Biomedical Research IMIBIC (2015).

3. Poster

Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D3.

VII Young Reseachers Meeting of the Maimónides Institute of Biomedical Research IMIBIC (2016).

4. Comunicación Flash

Improved SPE–LC–MS/MS platforms for determination of vitamin D and its metabolites.

XV meeting of GRASEQA (2016).

5. Comunicación Flash

Cromatografía bidimensional para la determinación de vitamina D y sus principales metabolitos, incluyendo la 3-epi-25(OH)D3, mediante espectrometría de masas

V Congreso Científico de Investigadores en Formación de la Universidad de Córdoba (2016).

6. Póster

Stability study for analysis of Vitamin D and its principal metabolites in serum samples.

VIII Young Reseachers Meeting of the Maimónides Institute of Biomedical Research IMIBIC (2017).

7. Póster

Serum vitamin D and breast cancer risk by pathological subtype

San Antonio breast cancer symposium (2017).

IV CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN DE LA UNIVERSIDAD DE CÓRDOBA

III CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN EN AGROALIMENTACIÓN

CREANDO REDES

CÓRDOBA, 18 Y 19 DE NOVIEMBRE DE 2014
EN EL RECTORADO DE LA UNIVERSIDAD DE CÓRDOBA
AVDA. MEDINA AZAHARA 5

APORTACIONES A LA METABOLÓMICA DE LA VITAMINA D PARA LA BÚSQUEDA DE BIOMARCADORES DE ENFERMEDADES

A. Mena-Bravo, F. Priego-Capote, MD. Luque de Castro

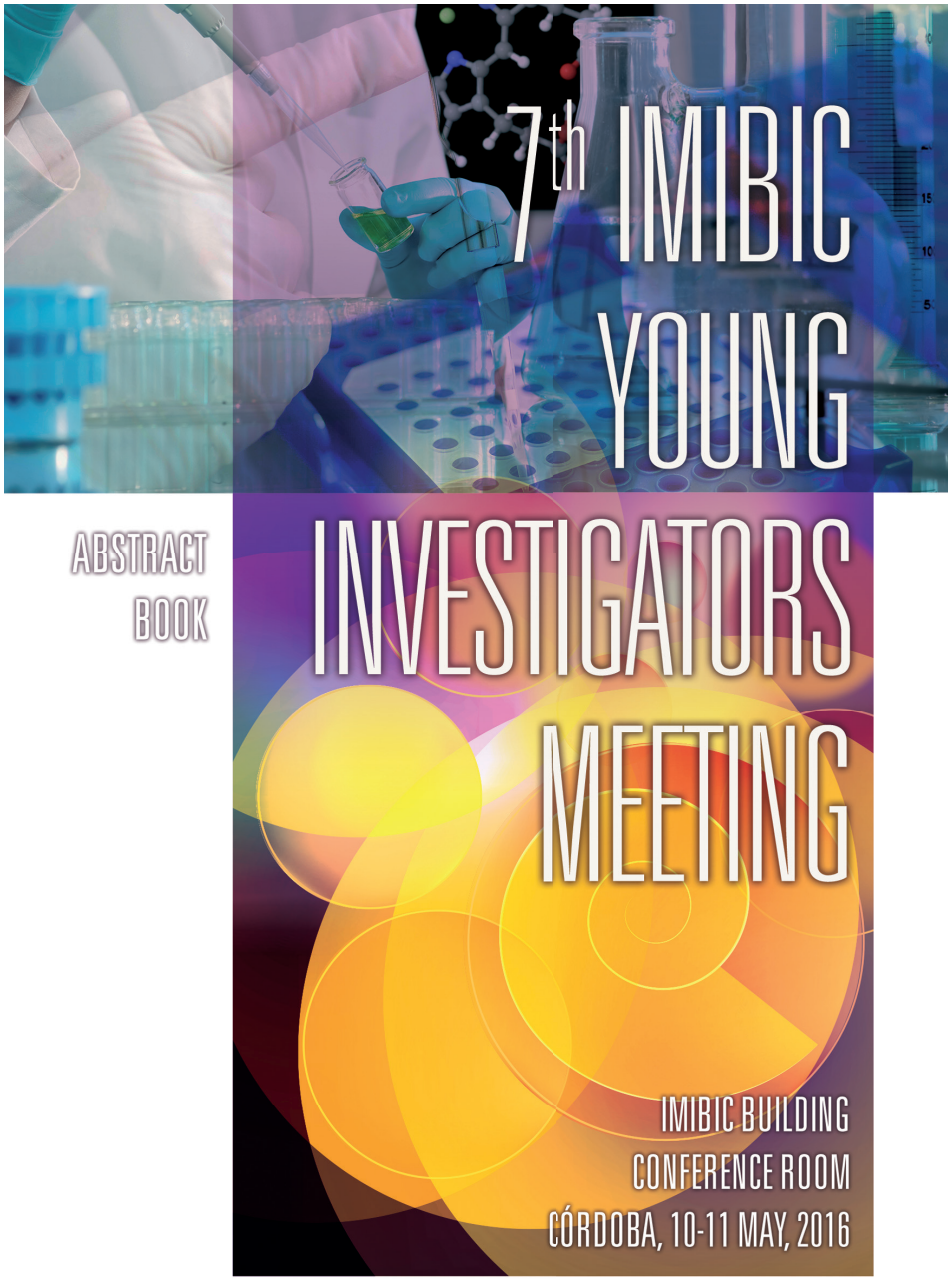
El estudio de las rutas metabólicas de la vitamina D (en sus dos formas, vitamina D_3 o colecalciferol y vitamina D_2 o ergocalciferol) ha puesto de manifiesto en múltiples investigaciones su capacidad de modificación en presencia de diferentes enfermedades. Su deficiencia se asocia con enfermedades óseas y cardiovasculares, diabetes, hipertensión y una amplia variedad de tipos de cáncer, entre las más importantes. Esta vitamina, inactiva biológicamente como tal, se hidroxila en el hígado formándose el metabolito 25-monohidroxivitamina D [25(OH)D] conocido como la forma circulante en sangre, y posteriormente se vuelve a hidroxilar en el riñón así como en diversos tejidos y células del sistema inmune formándose 1,25-dihidroxivitamina D [$1,25(OH)_2D$], que es la forma activa. Este metabolito se encuentra en concentraciones de pg/mL en el organismo humano y en animales, por lo que el estudio de su evolución y efecto es tremendamente complicado. No existen métodos fiables, suficientemente validados, para la determinación de calcitriol ($1,25(OH)_2D_3$) en fluidos biológicos. Los kits comerciales para este dihidroximetabolito están basados en inmunoensayo (radioinmunoensayo —RIA—, principalmente) y presentan baja especificidad debido, principalmente, a reacciones cruzadas en las que intervienen metabolitos como $24,25(OH)_2D_3$, producido durante el catabolismo de la vitamina D_3 en el riñón, que se encuentran en concentraciones muy superiores, del orden de los ng/mL, por lo que los resultados resultan menos fiables que al utilizar la cromatografía de líquidos acoplada a espectrometría de masas.


VI JORNADAS
DE JÓVENES
INVESTIGADORES
Edificio IMIBIC • Salón de actos • Córdoba, 4 y 5 de mayo de 2015
PROGRAMME
IMIBIC
INSTITUTO MAIMÓNIDES DE
INVESTIGACIÓN BIOMÉDICA
DE CÓRDOBA
JUNTA DE ANDALUCIA
UNIVERSIDAD D CÓRDOBA

Contributions to the analysis of vitamin D for the study of metabolic diseases

A. Mena-Bravo, F. Priego-Capote, M.D. Luque de Castro

The analysis of vitamin D status, with special emphasis on 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, is gaining interest in clinical studies due to the classical and non-classical effects attributed to this prohormone. In this research, two studies were developed: optimization of the method for quantitative analysis of vitamin D (both D2 and D3) and its main metabolites —monohydroxylated vitamin D (25-hydroxyvitamin D2 and 25-hydroxyvitamin D3) and dihydroxylated metabolites (1,25-dihydroxyvitamin D2, 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3) in human serum, and the influence of the two steps preceding determination (*viz.* sample collection and preparation) on the quantitative analysis of these analytes. The quantitative method is based on direct analysis of serum by an automated platform involving on-line coupling of a solid-phase extraction (SPE) workstation to a liquid chromatograph–tandem mass spectrometer. Detection of the seven analytes was carried out by the selected reaction monitoring (SRM) mode, and quantitative analysis was supported on the use of stable isotopic labeled internal standards (SIL-ISs). The method was externally validated according to the vitamin D External Quality Assurance Scheme (DEQAS) through the analysis of ten serum samples provided by this organism. The analytical features of the method support its applicability in nutritional and clinical studies targeted at elucidating the role of vitamin D metabolism. Two preparation approaches, deproteination and SPE, have been additionally evaluated in terms of sensitivity to delimit their application, thus establishing that detection of 1,25-dihydroxyvitamin D cannot be addressed by protein precipitation. Statistical analysis revealed that serum and plasma provided similar physiological levels for vitamin D3, 24,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3, while significantly different levels were obtained for 1,25-dihydroxyvitamin D3, always higher in plasma than in serum. Sample collection and treatment have proved to be significant in the analysis of vitamin D and its relevant metabolites.


7th IMIBIC
YOUNG
INVESTIGATORS
MEETING
ABSTRACT
BOOK
IMIBIC BUILDING
CONFERENCE ROOM
CÓRDOBA, 10-11 MAY, 2016

***P52*. Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D3.**

Authors: *A. Mena-Bravo, F. Priego-Capote, M. D. Luque de Castro.*

Group: GC21 Metabolomics Identification/Quantification of Bioactive Components.

The growing interest in the clinical effects of vitamin D and its metabolites has also increased the development of new methods for their determination. In addition to the well known role of vitamin D to maintain calcium homeostasis and prevent rickets and osteomalacia, other diseases such as pathogenesis of autoimmune diseases, cardiovascular disorders, infectious diseases, even inhibition of progression of breast, colon or pancreas cancer have been related to abnormal concentrations or ratios of vitamin D metabolites in humans. Recent studies have also shown the presence of the C3-epimer form of 25(OH)D3 in human serum, thus revealing that vitamin D can be metabolized through a parallel pathway, despite the enzyme responsible for 3-epimerization has not been identified. For these reasons, a method based on automated on-line solid phase extraction coupled to two-dimensional liquid chromatography with tandem mass spectrometry detection (SPE–2DLC–MS/MS) was developed for absolute quantitative profiling of vitamin D metabolites in human serum. Two-dimensional LC was configured with two complementary analytical columns, pentafluorophenyl (PFP) and C18 phases, for determination of 25 hydroxyvitamin D3 epimers and the rest of bioactive metabolites of vitamin D (D3 and D2) —25-hydroxyvitamin D2, 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D2 and 24,25-dihydroxyvitamin D3. Quantitative determination was supported on the use of a stable isotopic labelled internal standard for each analyte and the resulting method was validated by analysis of a standard reference material certified by the National Institute of Standards & Technology (NIST-972a) and 5 samples provided by the vitamin D External Quality Assurance Scheme (DEQAS). The limits of detection were between 9–90 pg/mL for the eight analytes, and precision, expressed as relative standard deviation, was lower than 11.6%. Two-dimensional LC has shown to be the key to discriminate between 25 hydroxyvitamin D3 epimers in a quantitative analysis also involving dihydroxyvitamin D metabolites.

XV REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

Almería, 30 de junio y 1 de julio de 2016

GRUPO REGIONAL ANDALUZ
SOCIEDAD ESPAÑOLA DE
QUÍMICA ANALÍTICA

UNIVERSIDAD DE ALMERÍA

CF-6

IMPROVED SPE–LC–MS/MS PLATFORMS FOR DETERMINATION OF VITAMIN D AND ITS METABOLITES

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The analysis of vitamin D status, with special emphasis on 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, is gaining interest in clinical studies due to the classical and new effects attributed to this prohormone. The developed research involved three steps: (1) the in-depth study of the two steps preceding determination (viz. sample collection and sample preparation) of vitamin D: both D2 and D3 and its main metabolites —monohydroxylated vitamin D (25-hydroxyvitamin D2 and 25-hydroxyvitamin D3) and dihydroxylated metabolites (1,25-dihydroxyvitamin D2, 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3)— in human serum [1]; (2) optimization of the analysis step of the target analytes [2]; and (3) improvement of the method to include quantification of the C3-epimer of 25(OH)D3 with the rest of bioactive metabolites of vitamin D.
(1) Statistical analysis revealed that serum and plasma provided similar physiological levels for vitamin D3, 24,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3, while significantly different levels were obtained for 1,25-dihydroxyvitamin D3, always higher in plasma than in serum. Two preparation approaches, deproteination and SPE, were evaluated in terms of sensitivity, thus establishing that detection of 1,25-dihydroxyvitamin D cannot be addressed by protein precipitation. Therefore, sample collection and treatment proved to be significant in the analysis of vitamin D and its relevant metabolites.
(2) The quantitative method was based on direct analysis of serum by an automated platform using the on-line coupling of a solid-phase extraction (SPE) workstation to a liquid chromatograph–tandem mass spectrometer. Detection of the seven analytes was carried out by the selected reaction monitoring (SRM) mode, and quantitative analysis was supported on the use of stable isotopic labeled internal standards (SIL-ISs).
(3) Finally, a new approach based on automated SPE coupled to two-dimensional liquid chromatographic with tandem mass spectrometry detection was developed for discrimination of 25 hydroxyvitamin D3 epimers. Two-dimensional LC was configured with two complementary analytical columns, pentafluorophenyl (PFP) and C18 phases, for determination of C3-epimer-25 hydroxyvitamin D3 and the rest of bioactive metabolites of vitamin D (D3 and D2) —25 hydroxyvitamin D3, 25-hydroxyvitamin D2, 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D2 and 24,25-dihydroxyvitamin D3. The method was externally validated according to the vitamin D External Quality Assurance Scheme (DEQAS) through the analysis of ten serum samples provided by this organism, and also by analysis of a standard reference material certified by the National Institute of Standards & Technology (NIST-972a).
The analytical features of the method support its applicability in nutritional and clinical studies targeted at elucidating the role of vitamin D metabolism.

[1] A. Mena-Bravo, F. Priego-Capote, M.D. Luque de Castro, Anal. Chim. Acta 879 (2015) 69–76
[2] A. Mena-Bravo, C. Ferreiro-Vera, F. Priego-Capote, M.A. Maestro, A.Mouriño, J.M. Quesada- Gómez, M.D. Luque de Castr, Clin. Chim. Acta 442 (2015) 6–12

VII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba, CREANDO REDES/Investiga y Comunica

UNIVERSIDAD D CÓRDOBA

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escuela internacional de doctorado en agroalimentación
SEDE CÓRDOBA

Córdoba, 6 y 7 de febrero de 2019

En el Rectorado de la Universidad de Córdoba

Avda. Medina Azahara 5

Cromatografía bidimensional para la determinación de Vitamina D y sus principales metabolitos, incluyendo la 3-epi-25(OH)D_3, mediante espectrometría de masas

A. Mena-Bravo, F. Priego-Capote, M. D. Luque de Castro

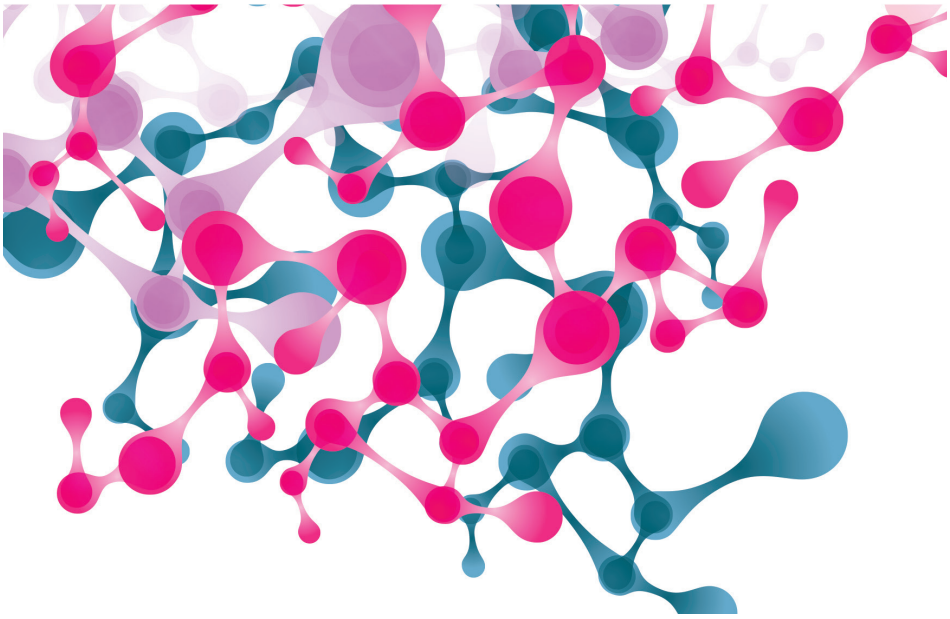
Summary

A novel two-dimensional liquid chromatographic method to quantify vitamin D (D_3 and D_2) and its main bioactive metabolites – monohydroxy metabolites (25 hydroxyvitamin D_3 and 25 hydroxyvitamin D_2, including the C3-epimer of 25 hydroxyvitamin D_3) and dihydroxy metabolites (1,25 dihydroxyvitamin D_3, 1,25 dihydroxyvitamin D_2 and 24,25 dihydroxyvitamin D_3) in human serum has been developed. The method is based on automated solid-phase extraction coupled to bidimensional chromatography, using pentafluorophenyl and C18 columns, followed by mass spectrometry determination. Quantitative analysis was supported on the use of stable isotopic labeled internal standard of each analyte and validated by Standard Reference Material provided by the National Institute of Standards & Technology (NIST–972a) and DEQAS (vitamin D External Quality Assurance Scheme). The limits of detection were between 9–90 pg/mL for the eight analytes, while precision, expressed as relative standard deviation, was below 11.6%. The bidimensional chromatography method has shown to be the appropriate configuration to determine the vitamin D forms and its main bioactive forms, including the C3-epimer of 25(OH)D_3.

8th IMIBIC YOUNG INVESTIGATORS MEETING

IMIBIC Building • Conference Room • Córdoba, 30-31 May, 2017

Abstract Book



P11. Stability study for analysis of Vitamin D and its principal metabolites in serum samples

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The role of vitamin D to prevent non-skeletal diseases such as cancer, autoimmune, cardiovascular or infectious disorders has been related to abnormal concentrations or ratios of vitamin D metabolites in humans. This connection has launched the interest in the clinical effects of vitamin D and its metabolites and also the development of new analytical methods with extra levels of sensitivity and selectivity. In this context, the stability of vitamin D metabolites and their lifetime in clinical samples have an essential relevance to ensure the quality of results. For this reason, a stability study of vitamin D3 and its metabolites - 25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ - in human serum has been developed by testing four different ways of samples storage (at room temperature, in a refrigerator at 4 ºC, and freezer at -20 ºC and -80 ºC) for two months. Additionally, the influence of freeze-thaw cycles on the concentration of the target analytes was evaluated. Serum samples were analyzed by an automated SPE-LC-MS/MS method that is participating in an external validation program termed DEQAS.

The four analytes experienced some differences in stability according to the storage conditions. As example, the circulating form, 25(OH)D_3, only provided a decrease of concentration under room temperature storage, while 24,25$(OH)_2D_3$ was only stable in the studied period after freezing at -80 ºC. Concerning freeze-thaw cycles, the two dihydroxymetabolites decreased their concentration after the third cycle; while the rest of analytes were stable after five freeze-thaw cycles. An additional test was based on the stability in lyophilized samples stored for two months, which revealed that the four analytes were stable under lyophilisation of the serum sample. This strategy could open a promising way to store serum/plasma for long periods prior to vitamin D analysis


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Carlos Arteaga, MD
Virginia Kaklamani, MD / DSc
Gary Chamness, PhD

2017 San Antonio Breast Cancer Symposium

Publication Number: P3-09-01

Title: Vitamin D and breast cancer risk by pathologic subtypes in Spain (MCC-Spain)

Marina Pollán[1,2], Virginia Lope[1,2], Adela Castelló[1,2], Antonio Mena-Bravo[3], Pilar Amiano[2,4], Nuria Aragonés[1,2], Tania Fernández-Villa[2,5], Marcela Guevara[2,6], Trinidad Dierssen[2,7], Guillermo Fernández-Tardón[2,8], Gemma Castaño-Vinyals[2,9], Rafael Marcos-Grajera[2,10], Víctor Moreno[2,11], Dolores Salas-Trejo[12], Marian Díaz-Santos[13], Manolis Kogevinas[2,9], Beatriz Pérez-Gómez[1,2] and Feliciano Priego-Capote[3]. [1]National Center for Epidemiology; Carlos III Institute of Health, Madrid, Spain; [2]Consortium for Biomedical Research in Epidemiology & Public Health (CIBERESP), Madrid, Spain; [3]University of Córdoba, Córdoba, Spain; [4]4Public Health Division of Gipuzkoa, BioDonostia Research Institute, Sam Sebastian, Guipuzcoa, Spain; [5]University of León, Institute of Biomedicine (IBIOMED), León, Spain; [6]Instituto de Salud Pública de Navarra, IdiSNA, Pamplona, Navarra, Spain; [7]University of Cantabria—IDIVAL, Santander, Cantabria, Spain; [8]IUOPA University of Oviedo, Oviedo, Asturias, Spain; [9]ISGlobal, Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain; [10]Epidemiology Unit & Girona Cancer Registry, Oncology Coordination Plan, Autonomous Government of Catalonia, Girona, Spain; [11]IDIBELL-Catalan Institute of Oncology, L'Hospitalet de Llobregat, Barcelona, Spain and [12]12Consellería de Sanidad Universal y Salud Pública, Generalitat Valenciana, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO), Valencia, Spain.

Body: Introduction: Vitamin D (VD) is involved in proliferation, apoptosis, differentiation, invasion and metastasis. Epidemiological studies show a clear protective effect of VD for colorectal cancer, while the evidence for breast cancer (BC) is less conclusive. Up to 50% of the population worldwide may have insufficient levels of 25-OH-vitamin D (25OHVD), the biomarker reflecting VD status. Our goal was to study the association between serum 25OHVD and BC risk by pathologic subtype in a subsample of BC cases/controls from a large case-control study in Spain.
Methods: MCC-Spain is a population-based multicase-control study on environmental and genetic factors (http://www.mccspain.org/). Here, we selected 558 BC cases who donated blood samples before starting chemotherapy, radiotherapy or hormonotherapy and 558 controls were frequently matched -by region, age and body mass index (BMI). 25OHVD was estimated by chromatograph-mass spectrometer in a sample of 200 µL serum. The effect (OR) of 25OHVD was estimated from mixed logistic regression models (random term = region), adjusted for age, BMI, menopausal status, reproductive factors family history of BC, previous biopsies, hypercholesterolemia, hormonal treatment, skin color, tobacco, physical activity and season of sampling. We used multinomial models -with the same co-factors- to estimate the association according to BC pathologic subtypes: a) luminal: ER+ or PR+ with HER2-, b) HER2+, and c) triple negative (TN): ER-, PR- and HER2- .
Results: 25OHVD levels were available in 546 cases and 558 controls. Mean 25OHVD was 48.2 nmol/L in controls and 43.6 in cases (P<0.01). Overall, BC risk decreased with increasing 25OHVD levels (OR per 10 nmol/L=0.88: P<0.001), with no differences in pre- and postmenopausal women. While similar ORs were found for luminal and HER2+ tumors (OR per 10 nmol/L= 0.89 and 0.88 respectively), a stronger effect was observed for TN (OR=0.64, P-heterogeneity: 0.039). No differences were observed according to TNM stage (P-heterogenity=0.706). Among BC cases, similar 25OHVD levels were observed in cases sampled in weeks 1&2 and weeks 3&4 after diagnosis, but 25OHVD level was lower among cases sampled > 1 month after diagnosis. A sensitivity analysis including only BC cases sampled in the first month showed a slightly lower effect (OR per 10 nmol/L=0.92; P=0.034), similar to the effect reported in meta-analyses of cohort studies. This analysis confirmed the stronger association found for TN tumors (OR= 0.53, P=0.001; P-heterogeneity=0.012). Finally the dose-response analysis showed a downward (protective) effect up to concentrations around 90 nmol/L, from which the risk increased. However, only 3% of women had levels over 90 nmol/L.
Conclusions: To our knowledge, this is the 1st study providing information on the effect of VD by BC subtypes. Our results show a clear protective effect, particularly against TN tumors.
Funding: Spain's Health Research Fund (*Fondo de Investigación Sanitaria* - FIS PI12/00488); Ministry of Health (EC11-273).

ABREVIATURAS

ABBREVIATIONS

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D.

$1,25(OH)_2D_2$: 1,25-dihydroxyvitamin D_2.

$1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3.

$24,25(OH)_3D$: 24,25-dihydroxyvitamin D.

$24,25(OH)_2D_3$: 24,25-dihydroxyvitamin D_3.

25(OH)D: 25-hydroxyvitamin D.

$25(OH)D_2$: 25-hydroxyvitamin D_2.

$25(OH)D_3$: 25-hydroxyvitamin D_3.

3-epi-$25(OH)D_3$: 3-epi-25-hydroxyvitamin D_3.

ACE: automatic cartridge exchange.

ACN: acetonitrile.

ALTM: all-laboratory trimmed mean.

ANOVA: one-way analysis of variance.

APCI: atmospheric pressure chemical ionization.

APPI: atmospheric pressure photoionization.

BMI: body mass index.

CE: capillary electrophoresis.

CLIA: chemiluminescence immunoassays.

CPB: competitive protein binding assay.

CYP24: cytochrome P450-1,25-dihydroxyvitamin D_3 24-hydroxylase.

CYP27B1: cytochrome P450-25-hydroxyvitamin D3 1alpha-hydroxylase.

DAD: diode array detector.

DBP: vitamin D binding protein.

DBS: dried blood spots.

DEQAS: vitamin D External Quality Assurance Scheme.

DIMS: direct infusion mass spectrometry.

EICs: extracted ion chromatograms.

ELISA: enzyme linked immune sorbent Assay.

ESI+: electrospray ionization in positive mode.

FI: flow-injection.

FTICR: Fourier transform ion cyclotron resonance.

GC: gas chromatography.

HPDs: high-pressure syringe dispensers.

IDS: immuno diagnostic systems.

IS: internal standard

LC: liquid chromatography.

LC–MS/MS: liquid chromatography–tandem mass spectrometry.

LLE: liquid–liquid extraction.

LOD: limit of detection.

LOQ: limit of quantitation.

MEs: matrix effects.

MRM: multiple reaction monitoring.

MS: mass spectrometry.

NIST: National Institute of Standards & Technology.

NMR: nuclear magnetic resonance.

OT: Orbitrap.

PFP: pentafluorophenyl.

PP: protein precipitation.

PTAD: 4-phenyl-1,2,4-triazolie-3,5-dione.

Q: single quadrupole.

QC: quality control.

QIT: quadrupole ion trap.

QqQ: triple quadrupole.

QTOF: quadrupole time of flight.

RIA: radioinmunoassay.

RP–LC: reversed-phase liquid chromatography.

RRB: radioreceptor binding.

RSD: relative standard deviation.

SAM: standard addition method.

SF: supercritical fluid.

SFC: supercritical fluid chromatography.

SIL-IS: stable isotopic labeled internal standards.

SOPs: standard operating procedures.

SPE: solid phase extraction.

SRM: selected reaction monitoring.

TOF: time of flight.

VDES: vitamin D endocrine system.

VDR: vitamin D receptor.

WHR: waist-to-hip ratio